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Effects of Omega-3 Fatty Acids and Hypoxia on Progesterone Biosynthesis and Mitochondrial Morphology in the Corpus Luteum

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EFFECTS OF OMEGA-3 FATTY ACIDS AND HYPOXIA ON PROGESTERONE BIOSYNTHESIS AND MITOCHONDRIAL MORPHOLOGY IN THE CORPUS LUTEUM

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ABSTRACT

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The corpus luteum is a transient steroidogenic endocrine gland that forms from the remnants of the ovulatory follicle. This gland is responsible for the synthesis and secretion of the hormone progesterone, which is critical for the establishment and maintenance of pregnancy. In a non-pregnant cow, PGF2a is released from the uterus to regress the corpus luteum. However, in the case of pregnancy, the embryo must mitigate PGF2a synthesis to prevent corpus luteum regression. If the embryonic signal is either too late or too weak, uterine PGF2a secretion may result in corpus luteum regression and termination of pregnancy. Omega-3 fatty acids from fish byproduct may provide a nutraceutical approach to reduce luteal sensitivity to PGF2a, which provides a potential solution to early embryo loss.

Recent data show that incorporation of omega-3 fatty acids into luteal tissue reduces sensitivity to PGF2a. However, mRNA for key genes that regulate progesterone synthesis were significantly decreased. It was hypothesized that omega-3 fatty acids may allow for a rebound in mRNA or adequate luteal cell protein abundance allowing for synthesis and secretion of progesterone following intrauterine infusions of PGF2a. Additionally, it was hypothesized that omega-3 fatty acids from fish byproduct affect both lipid droplet accumulation and size in bovine luteal tissue and cells, as well as

mitochondrial dynamics, providing a lipid rich and healthy environment for steroid synthesis following PGF2a infusions and a low oxygen environment.

Data from the present study show that fish oil supplementation was luteal protective in response to PGF2a infusion. Steady-state mRNA for key genes that regulate steroidogenesis was significantly increased at 48 h post PGF2a infusion as compared to animals receiving vegetable oil supplementation. Additionally, fish oil supplementation *in vitro* improved luteal cell mitochondrial morphology as compared to control cells in a low oxygen environment.

In conclusion, supplementation of fish oil improved luteal function during PGF2a infusion and hypoxia. Outcomes from these studies may allow for development of novel feeding strategies to reduce luteal sensitivity during maternal recognition of pregnancy resulting in improved reproductive efficiency in cattle.

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Bovine Reproductive Cycle

The bovine reproductive cycle is approximately 21 days in length and includes estrus, metestrus, diestrus and proestrus (Fields and Fields, 1996). Figure 1 shows relative hormone concentrations during different phases of the reproductive cycle.

Bovine Reproductive Cycle - Estrus

The cycle begins with estrus which is a short period at the beginning of the cycle (Senger, 1997). This stage of the cycle is defined as day 0 and generally referred to as "standing heat". Estrus behavior lasts around 18 hours. It is during this during this short window of time that the cow will allow other cows or bulls to mount. A dominant follicle growing on the ovary releases a large amount of estradiol into the blood. Estradiol, a steroid hormone, acts on a complex neuronal circuit that ultimately allows for a surge of GnRH (gonadotropin-releasing hormone) from the hypothalamus. GnRH neurons synapse at the median eminence and GnRH is secreted into the portal vessels (Kumar and Sharma, 2014). GnRH is transported to the anterior pituitary and binds to high affinity receptors expressed on the gonadotrophs that trigger a release of LH (luteinizing hormone) and FSH (follicle-stimulating hormone). LH will allow the dominant follicle to ovulate, releasing the oocyte from the follicle into the oviduct. Ovulation generally

occurs 24 to 30 h after onset of estrus. Estradiol also targets neurons of the limbic system to drive estrus behavior.

Bovine Reproductive Cycle - Metestrus

Metestrus is days 1 to 5 of the cycle. During this phase of the cycle, LH reprograms cells of the ruptured follicle to undergo a transition of proliferation and differentiation into the corpus hemorrhagicum. This is the early development of the corpus luteum. During metestrus, progesterone levels begin to become detectable in the blood of the animal. As the corpus hemorrhagicum undergoes significant transition, the theca and granulosa cells of the ovary (which are the follicular steroidogenic cells that synthesize androgens and estradiol) become small and large luteal cells, respectively, referred to as luteinization (Alila and Hansel, 1984). In order to sustain rapid cell growth of the developing corpus luteum, angiogenesis also occurs during this period (Fields and Fields, 1996).

Bovine Reproductive Cycle - Diestrus

Diestrus is approximately days 6 to 16 of the cycle. During this period of the cycle, a mature corpus luteum is present on the ovulated ovary. Copious amounts of progesterone are secreted from the corpus luteum and is therefore easily detected in the blood at this phase, normally between 3-10 ng/mL (Sirois and Fortune, 1990; Baird et al., 1976). Additionally, the cells of the corpus luteum continue to grow in abundance, which is essential to increasing progesterone output. If a pregnancy were to occur, the corpus luteum would be advanced enough to produce adequate progesterone to maintain the pregnancy (Forde et al., 2009). Progesterone acts on the uterine endometrial glands to increase histotroph secretion, proteins that support early embryo development prior to

implantation. Additionally, the trophoblastic cells of the embryo release interferon-tau (IFN-t). This IFN-t signal prevents release of uterine prostaglandin (PG)F2a and thereby prevents regression of the corpus luteum. During the estrous cycle, the cow will exhibit 2 to 3 waves of follicular growth. The wave of follicular growth that occurs during late diestrus will lead to an increase in estradiol. It is this increase in estradiol that is responsible for an increase in uterine endometrial oxytocin receptor number that results in pulsatile secretion of PGF2a in the non-pregnant cow.

Bovine Reproductive Cycle - Proestrus

Days 17 to 21 of the cycle is defined as proestrus (Senger, 1997). During proestrus, regression of the corpus luteum occurs in the non-pregnant cow in a process known as luteolysis (Hansel, 1966). To initiate luteolysis, PGF2a is released from the uterus in a series of pulses that begin on days 16 to 17 of the cycle (Hansel, 1966). As the corpus luteum regresses, progesterone levels begin to decline. With the corpus luteum no longer present, blood levels of progesterone remain lower than 1 ng/mL (Assey et al., 1993). With minimal progesterone negative feedback on the hypothalamus and anterior pituitary gland, pulsatile secretion of GnRH increases in frequency (Moenter et al., 1992). This stimulates follicular growth resulting in increased estradiol secretion. Once estradiol reaches a threshold point, a surge of GnRH is released from the hypothalamus which acts on the anterior pituitary to signal for a surge of LH release (Moenter et al., 1992). This LH surge will ovulate the dominant follicle, which will grow into a new corpus luteum, and initiation of a new cycle.

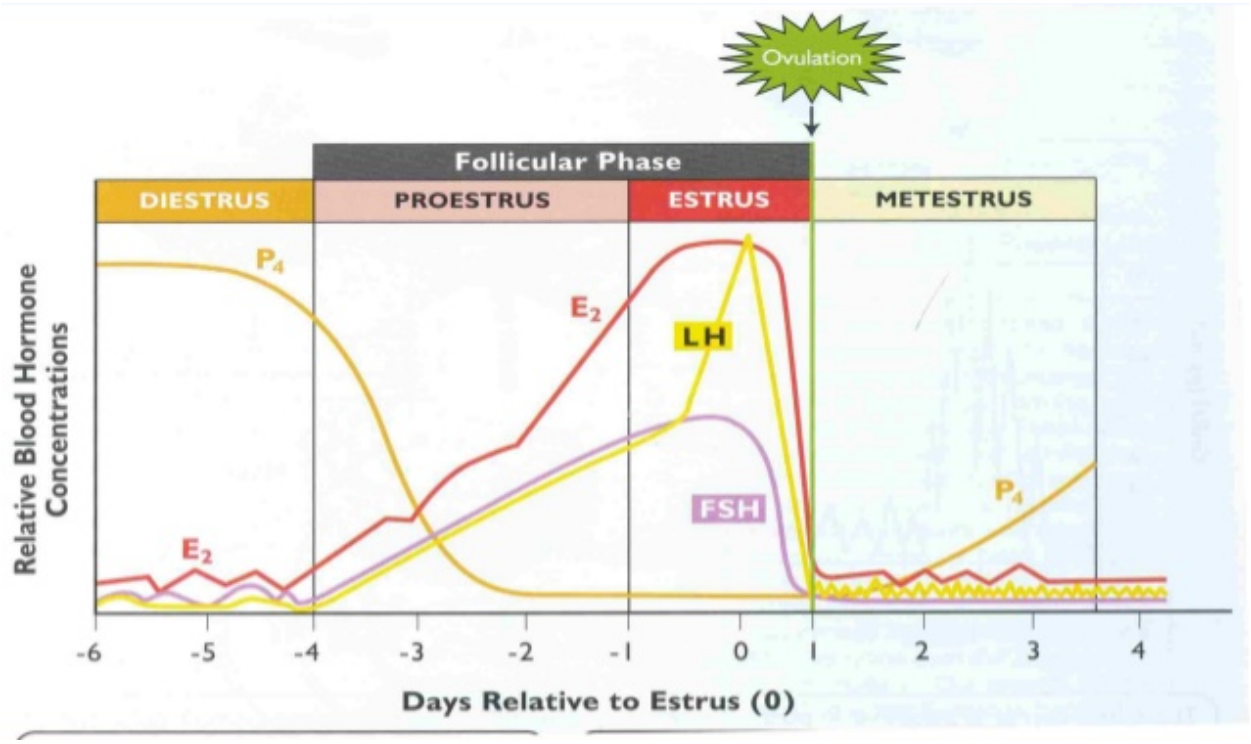

Figure 1: A schematic diagram showing the bovine estrous cycle, indicating phases of the cycle and relative hormone concentration. Adapted from Senger, 1997.

Maternal Recognition of Pregnancy

Research shows that approximately 30% of bovine embryos die within the first few weeks of early gestation (Dunne et al., 2000), resulting in a large loss of potentially viable offspring (Berg et al., 2010). Loss of pregnancy costs the United States beef and dairy industry millions of dollars annually in decreased milk and beef production. Factors that can lead to early embryonic loss include failure of placental attachment, chromosomal abnormality, and failure in maternal recognition of pregnancy (Thatcher et al., 1994). During maternal recognition of pregnancy (days 16 to 23 following mating), the embryo must send a signal to halt reproductive cycling and ensure development. In a non-pregnant animal, the uterus releases PGF2a (Nancarrow et al., 1973), which is the natural luteolysin for ruminant animals. This signal will lead to the regression of the

corpus luteum – i.e. a decrease in progesterone and involution of the gland (Roberts et al., 1996). During early pregnancy, the trophoblastic cells of the embryo secrete IFN-t, which inhibits synthesis of PGF2a, thereby protecting the corpus luteum and allowing pregnancy to continue (Bazer et al., 2013). It is postulated that these cells are unable to secrete an IFN-t signal strong enough, or the signal is delayed, it can result in PGF2a and regression of the corpus luteum. As a result, the pregnancy is lost.

Pulsatile secretion of PGF2a is mediated by oxytocin. The source of oxytocin is hypothesized to be from the posterior pituitary and/or the corpus luteum (Fields et al., 1983). As estradiol concentration increases during late diestrus, synthesis of uterine oxytocin receptors subsequently increases (Perumamthadathil et al., 2014). Oxytocin binds to these receptors and stimulates generation and secretion of PGF2a from the epithelial cells of the uterine endometrium (Burns et al., 1998). The function of IFN-t is to inhibit expression of estrogen and oxytocin receptors on the uterine endometrium during early pregnancy. By inhibiting receptor expression, oxytocin mediated PGF2a release is inhibited.

There are several ways to mitigate early pregnancy loss in cattle. (Diskin et al., 2016). Administration of intramuscular injections of IFN-t following mating can be one approach (Meyer et al., 1996). However, this is impractical as it requires injection of IFN-t at least 2 times daily from days 14 to 17 of pregnancy (Roberts et al., 2009). Furthermore, in the United States beef and dairy industry, animals are managed in large herds, making this both too labor intensive, as well as financially impractical. Rather than treating the animal with additional IFN-t, another approach is to reduce luteal sensitivity to PGF2a following mating. This would negate the problem of a late IFN-t signal from

the embryo. Omega-3 polyunsaturated fatty acids have been shown to reduce sensitivity to PGF2a (Plewes et al., 2018). By supplementing with omega-3 fatty acids, this potentially provides the developing conceptus more time to adequately produce enough IFN-t to inhibit PGF2a synthesis and protect the corpus luteum. By doing so, this would preserve maternal recognition of pregnancy, and the embryo is protected.

Omega-3 fatty acids are found in a number of dietary supplementations (Doughman et al., 2007). This option is far more cost effective than IFN-t injection, given that the cost of fish oil is significantly less than pharmaceutical treatments. Dietary supplementation of omega-3 fatty acids is a non-invasive method that would reduce stress on the animal. Through consumption in diet, omega-3 fatty acids can be incorporated into reproductive tissues of the animal thereby reducing luteal sensitivity to PGF2a and potentially improving reproductive success (Plewes et al., 2018).

Physiology of the Corpus Luteum

The corpus luteum is a transient endocrine gland that grows from the remnants of the ovulatory follicle. This gland is responsible for the secretion of progesterone, a steroid hormone essential for both the establishment and maintenance of mammalian pregnancy (Corner et al., 1937). This gland was first discovered by Regner de Graaf in 1672 and subsequently named the gland “corpus luteum” in 1689 by Marcello Malpighi due to its yellow color by nature. In 1898, Louis-Auguste Prenant and Gustav Born furthered demonstrated that the corpus luteum was an organ of internal secretion, also known as an endocrine gland (Prenant et al., 1898). Endocrine glands secrete chemical messenger signals known as hormones directly into the bloodstream allowing for distant signaling.

It was first reported in rabbits that the corpus luteum was essential to pregnancy (as reviewed by Gadsby et al., 1984). Removing corpora lutea from pregnant rabbits resulted in loss of embryos and termination of the pregnancy. When luteal fluid was injected back into animals that had no CL, pregnancy was retained. This discovery showed there was a chemical signal from the corpus luteum that is involved in pregnancy and embryo development. In 1934, the chemical was extracted from the corpus luteum, purified, chemically analyzed and subsequently named progesterone (Allen and Goetsh, 1936; Corner et al., 1937).

During the luteal phase of the reproductive cycle, progesterone is secreted in high amounts that targets and prepares the uterus for an ensuing pregnancy (Corner et al., 1937). Progesterone is transported through the bloodstream by carrier proteins, due to progesterone being a lipid-derived steroid hormone. Eventually this hormone reaches its target tissue and subsequently binds to its respective progesterone receptor (Graham et al., 1997). A major target tissue of progesterone is the uterus (Graham et al., 1997). Progesterone prepares the uterus for pregnancy by:

1. converting the uterine endometrium to the secretory phase, in which it secretes histotrophs for early embryo development (Di Renzo et al., 2016).
2. decreasing the maternal immune response to the developing embryo, allowing for successful pregnancy,
3. reducing myometrial contractions during this period.

Progesterone also plays a key role in negatively inhibiting gonadotropin-releasing hormone (GnRH) and subsequently blocks secretion and release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Girmus et al., 1991). Inhibition

of both LH and FSH is characteristic of the luteal phase of the reproductive cycle (Girmus et al., 1991).

The Corpus Luteum

The corpus luteum develops from the ovulatory follicle and is comprised of 4 major cell types (O'Shea et al., 1989; Alila and Hansel, 1984).

Luteal Cells

The first of these cells are small and large luteal cells, occupying the largest volume of gland mass. These cells originate from the theca and granulosa cells of the follicle, respectively (Murphy et al., 2000). The small and large luteal cells are often known as the steroidogenic cells of the corpus luteum (Fitz et al., 1982). Despite being similar in steroidogenic nature, small and large luteal cells are highly differentiated and large luteal cells alone make up roughly 40% of the total volume of the corpus luteum (Weber et al., 1987).

Large luteal cells have a higher capacity for steroid production. Large luteal cells have an average diameter of 38 μm, compared to a diameter of 17 μm in small luteal cells (Yoshioka et al., 2013). Proportionally, large luteal cells account for 1 out of every 7.6 small luteal cells, meaning small luteal cells are considerably more abundant in the corpus luteum (Alila and Hansel, 1984). Using centrifugal elutriation to separate cells by density, isolated ovine small and large luteal cells showed differing mechanisms for progesterone synthesis and production (Fitz et al., 1982). Basal production of progesterone differs greatly when comparing large and small luteal cells. Large luteal cells on average produce 20 times the amount of progesterone compared to small luteal cells (Weber et al., 1987). Likewise, LH-induced progesterone production differs

between cell type. With addition of LH, small luteal cells are shown to have a considerable response with a significant increase in progesterone output (20 to 40 times increase). Large luteal cells show only a modest response (2 to 4 times increase; Weber et al., 1987). Given a high enough concentration of LH (> 100 ng/ml), bovine large luteal cells can exhibit a greater response in progesterone output, approximately doubled (Stocco et al., 2007).

In a mid-cycle corpus luteum, small and large luteal cells may act synergistically to enhance progesterone production. This synergy loses its effectiveness during corpus luteum regression as both small and large luteal cells begin to become apoptotic. As one cell type begins to lose its function, cell to cell interactions are lost and synergy is compromised. Large luteal cells have been shown to bind more PGF2a or PGE2 than small luteal cells (Hansel et al., 1991). As a result, large luteal cells have a greater number of prostaglandin receptors compared to small luteal cells (Hansel et al., 1987).

Both small and large luteal cells have similar internal organelles. Both cell types contain smooth endoplasmic reticulum, mitochondria, Golgi, and lipid droplets. A major distinguishing difference between large and small luteal cells is the presence of electron-dense granules in the cytoplasm of granulosa-derived large cells, as opposed to theca-derived small cells (O'Shea et al., 1989).

In addition to morphological differences, small and large luteal cells differ by both protein and gene expression (O'Shea et al., 1989). Large and small luteal cells express relatively equal amount of CDH12 (Cadherin-12). Additionally, FSH receptor expression does not differ between the two cell types (Romereim et al., 2017). Large luteal cells express greater numbers of oxytocin receptors (McArdle et al., 1989). PTHLH

(parathyroid hormone-like hormone) is significantly increased in large luteal cells compared to small cells. LHCGR (LH receptor) expression is greatly increased in small luteal cells compared to large luteal cells (Romereim et al., 2017). In the ovine, oxytocin production is highly elevated in large luteal cells compared to small (Rodgers et al., 1983). Oxytocin output of large luteal cells is on average 0.70 fg/cell, which accounts for 30 times the oxytocin production of a small luteal cell. There, it appears that the primary source of oxytocin in the ovine corpus luteum is produced via large luteal cells.

Additional Cell Types

While steroidogenic cells comprise a large portion of the corpus luteum, endothelial cells and fibroblasts make up most of the rest of the cell composition (Davis et al., 2003). Endothelial cells account for roughly 14% of the total volume of the corpus luteum (O'Shea et al., 1989) that is necessary for vasculature in the corpus luteum. Although steroidogenic cells account for more volume, endothelial cells account for the greatest number (O'Shea et al., 1989). Fibroblasts account for 7% of the total volume of the gland (Irving-Rodgers et al., 2006). Given that most of the volume is occupied by small and large luteal cells, this leaves roughly 10% of the volume of the corpus luteum for additional cell types such as immune cells. In the corpus luteum, collagen comprises a large amount of the extracellular matrix (Irving-Rodgers et al., 2006). Collagen type 1 is a fibrillar collagen that aids in strength and durability of tissue. Collage type 1 subunits can be found in the bovine corpus luteum and are found in excess during early corpus luteum development (Helen et al., 2006). Basal lamina tissue surrounds the endothelial cells and vasculature (Irving-Rodgers et al., 2006). Collagen type 4 was found in

endothelial basal laminas contained in the bovine corpus luteum (Irving-Rodgers et al., 2006).

Immune cells are likely recruited during the period of ovulation, around day 0. Prior to ovulation, macrophages are present in the ovary and may play a role in follicular maturation (Bauer et al., 2001). After the LH surge, macrophages and other immune cell populations increase (Kfir et al., 2018). Recruitment of immune cells to the mature corpus luteum requires immune and endothelial cell interaction (Walusimbi et al., 2013). T lymphocytes have been shown to aid in progesterone production in human and rat, but not bovine (Bauer et al., 2001). Additionally, neutrophils and eosinophils have been shown to play a role in luteal angiogenesis in bovine (Bauer et al., 2001). Reducing the number of eosinophils in the developing bovine corpus luteum results in decreased plasma progesterone and decreased vascular endothelial growth factor, which in turn affects angiogenesis.

Corpus Luteum Vasculature

The corpus luteum is one of the most vascularized tissues in the body (Nishimura et al., 2010) and in order to support such rapid cell growth of the corpus luteum over the course of only a few days, angiogenic factors must be highly expressed. These angiogenic factors must be highly expressed during corpus luteum development and during the luteal phase to allow for adequate blood flow. Cell cycle promoters (cyclins, cyclin-dependent kinases) must also be upregulated in order to increase cell number (Kowalewski et al., 2015; Jiang et al., 2011). The corpus luteum develops during hypoxia due to low oxygen provision from a lack of vasculature during early corpus luteum formation (Fadhillah et al., 2014). As a result of hypoxia, hypoxic inducible factor 1

(HIF1) is highly expressed (Fadhillah et al., 2017). A commonly known target of HIF1 includes vascular endothelial growth factor (VEGF; Fadhillah et al., 2017). Insulin-like growth factor (IGF) and gonadotropins such as LH and FSH have also been known to upregulate VEGF in a hypoxic environment. VEGF is the primary driver of angiogenesis. During corpus luteum formation, HIF1A mRNA expression is higher during early and mid-stage corpus luteum development as opposed to corpus luteum regression (Nishimura et al., 2010). Mitogen activated protein (MAP) kinase is also highly upregulated during this time and will further aid in cell proliferation as well as gene expression. VEGF will be upregulated in response to MAP kinase activation (Nishimura et al., 2019).

Luteal Progesterone Synthesis

Hormones are classified as either peptide/protein, amino acid derived, steroid or fatty acid derived (Rawn et al., 1989). Progesterone is a steroid hormone and the precursor to all steroid hormones is cholesterol. There are several sourced of cholesterol that can be utilized by steroidogenic cells. This can be synthesized *de novo* in all mammals or derived from the diet through consumption of animal products (Carr et al., 1980).

The most common form of cholesterol intake is low-density lipoprotein (LDL) and high-density lipoprotein (HDL; Hu et al., 2010). LDL is shuttled into the cell through the low-density lipoprotein receptor (LDLR) and HDL is shuttled into the cell through the scavenger B1 receptor (Feingold and Grunfeld, 2018). In addition, steroidogenic cells often store cholesterol intracellularly as lipid droplets (LDs) which are organelles used for lipid storage, composed primarily of cholesterol esters and triglycerides (Zhang et al.,

2010). Lipoproteins bind to their respective receptors for intracellular entry and cholesterol from lipoproteins is commonly stored as LDs or shuttled directly into the mitochondria for steroid synthesis (Zhang et al., 2010). All cell types synthesize cholesterol *de novo*. During *de novo* synthesis, cholesterol is synthesized from acetyl CoA (Carr et al., 1980). Acetyl CoA is then converted to mevalonate (Carr et al., 1980). HMG CoA reductase catalyzes the rate limiting step in cholesterol synthesis (Carr et al., 1980). Despite the ability to synthesize cholesterol *de novo*, steroidogenic cells do not frequently use this metabolic pathway. The last form of cholesterol intake is from the plasma membrane (Zhang et al., 2010). The cell membrane is largely composed of glycerophospholipids, sphingolipids and cholesterol. Cells are able to obtain cholesterol from the membrane through use of various phospholipases (Litvinov et al., 2018).

Most cholesterol used for steroid biosynthesis is either shuttled directly into the mitochondria, or from lipid droplets in the form of cholesterol ester (Rekawiecki et al., 2008). Sterol O-acyltransferase (ACAT) enzyme forms cholesterol esters from free cholesterol. Hormone sensitive lipase (HSL) is necessary to hydrolyze cholesterol esters allowing for mobilization of cholesterol (Yeaman et al., 1994). Activity of HSL is regulated by protein kinases (Yeaman et al., 1994). Cholesterol from either LDs or directly from lipoproteins is then shuttled into the mitochondria by steroidogenic acute regulatory protein (StAR). StAR is a major protein in a complex involved in cholesterol shuttling. Cholesterol shuttling is the rate limiting step of steroid biosynthesis. As such, many cellular pathways are coupled to increase StAR protein and activity. LH works through the LHCGR receptor to increase adenylate cyclase (AC) and activate cAMP (Allen et al., 2016). This increases protein kinase A (PKA) expression. PKA activity and

expression both subsequently increase StAR expression. Additionally, the plant compound forskolin is a powerful activator of AC and increases both cAMP and PKA expression, as well as being known to increase both StAR expression and progesterone production. Dibutyryl cyclic AMP (dBcAMP) is a way to bypass activation of AC and directly increase PKA expression. Furthermore, the use of StAR protein can be completely avoided by addition of a cholesterol derivative such as 22R-hydroxycholesterol. Once cholesterol is shuttled into the mitochondrion as regulated by StAR, it is then converted to the first intermediate, pregnenolone. P450scc, side chain cleavage enzyme (CYP11A1) is responsible for converting cholesterol to pregnenolone. During this conversion the cholesterol side chain is cleaved, converting the 27-carbon cholesterol into the 21-carbon pregnenolone. Despite pregnenolone being synthesized within the mitochondrion, majority of progesterone is synthesized within the smooth endoplasmic reticulum.

There is debate as to how this intermediate is transported to the ER for progesterone synthesis. It is postulated that the mitochondria and ER come in contact that forms a mitochondrial-associated membrane complex (van Vliet et al., 2018). In this model, the ER makes a physical connection with the mitochondria. This is tightly regulated through a series of voltage dependent anion channels (VDAC) and ATAD3, an AAA ATPase (Shoshan-Barmatz et al., 2010; Issop et al., 2015). Pregnenolone could also relocate to the ER by diffusion through the mitochondrial membrane, and towards the ER. At the smooth ER, the enzyme 3β-Hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-HSD) converts pregnanolone to progesterone. Once fully synthesized, progesterone diffuses out of the cell into the surrounding bloodstream. Figure 2 shows a simplified

diagram of progesterone biosynthesis. Since steroids are lipid soluble, it requires use of albumin or other carrier protein for transport in the blood plasma. Sex hormone-binding globulin (SHBG) is also bound to progesterone for blood transport (Hammond et al., 2016). Once progesterone is in the blood plasma, it is carried to its target tissue (Skinner et al., 1998), which is commonly the uterine endometrium (Harold et al., 1968; Salehnia et al., 2013). Figure 3 shows progesterone binding to its respective receptor, activating downstream targets. In the uterus, progesterone blocks myometrial smooth muscle contractions by preventing estradiol from binding to alpha-adrenergic receptors found in the uterine environment, which would stimulate contractions (Bottari et al., 1983; Stevenson et al., 2016). Progesterone is also known to target the brain and mammary glands (Rekawiecki et al., 2008). Additionally, progesterone has anti apoptotic effects (Rueda et al., 2000). Fas ligand is a common apoptotic receptor found on bovine cells of the corpus luteum. Progesterone is involved with the bcl-2 protein family (Niswender et al., 2000). As such, progesterone aids in inhibiting Fas ligand, as well as caspase-3 expression and activation (Okuda et al., 2004).

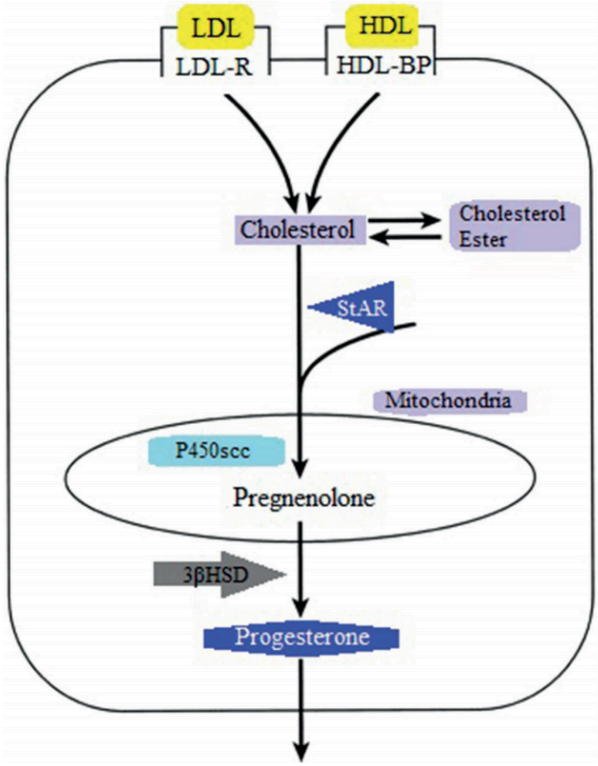

Figure 2: Representative model showing progesterone biosynthesis in a bovine luteal cell. Image adapted from Kuru et al., 2018.

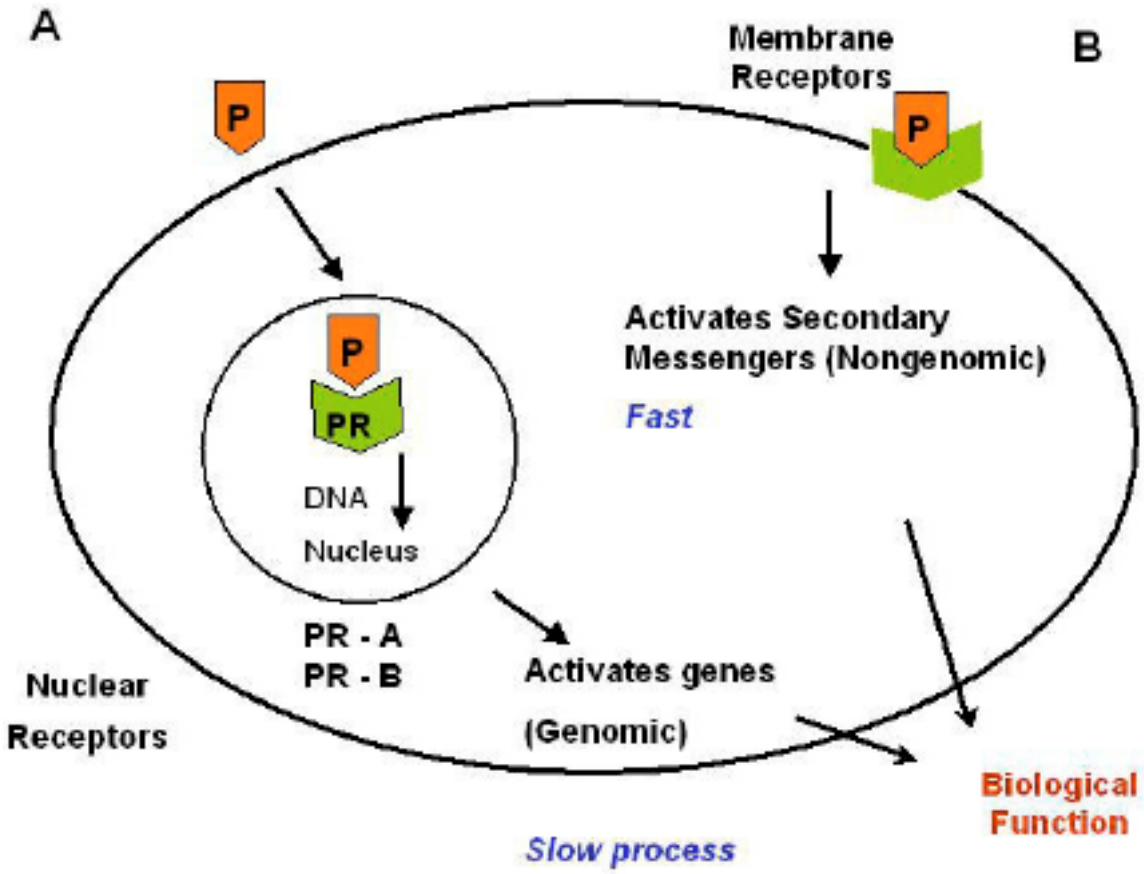

Figure 3: Mechanism of action showing progesterone and progesterone receptor effects on target cell. Image adapted from Gadkar-Sable et al., 2005.

Luteolysis

If no pregnancy occurs following ovulation, the corpus luteum must regress for the reproductive cycle to repeat, defined as luteal regression, or luteolysis (Bowen-Shauver et al., 2003). Late in the cycle (days 15-18), PGF2a is released from the uterus to initiate corpus luteum regression in a series of pulses (Silvia et al., 1991; Nancarrow et al., 1973; Peterson et al., 1975; Shirasuna et al., 2004).

There are two independent events that occur during luteolysis, functional regression and structural regression. Functional regression corresponds to the dramatic decrease in progesterone secretion from the corpus luteum. Serum progesterone decline is detectable with 6-8 hours following PGF2a uterine pulses. During PGF2a-induced

lutoelysis, gene expression and activity for key proteins required for progesterone synthesis such as LDLR, StAR, CYP11A1, 3B-HSD and HSL are significantly decreased. Following repeated pulses of PGF2a, progesterone decreases to < 1 ng/ml, indicating functional regression (Assey et al., 1993), as shown by gene expression for StAR, CYP11A1, NR5A1, LHCGR and PTGFR. Average mRNA for all steroidogenic genes decreases 2-fold following PGF2a treatment post 12 hours. By 18 hours, gene expression for steroidogenic proteins decreases 4-fold. Protein expression for StAR, CYP11A1 and 3B-HSD is reduced significantly following repeated doses of PGF2a (Rodgers et al., 1995). PGF2a anti-steroidogenic actions are mediated by PKC signaling (Wiltbank et al., 1990). Through PKC signaling and decrease in gene expression, progesterone production is reduced significantly. Cholesterol transport for steroid production is downregulated due to decrease in mRNA for LDLR and HSL. Cholesterol shuttling for steroid synthesis through the mitochondria is decreased due to low mRNA levels for StAR, as well as low phosphorylation levels and CYP11A1 in response to PGF2a (Atli et al., 2012). A decrease in mRNA expression for NR5A1 encodes for Steroidogenic factor 1 (SF-1). SF-1 is a transcription factor which is responsible for expression of steroid hydroxylases. These steroid hydroxylases are important for farther downstream steroid biosynthesis, such as conversion of progesterone to steroids like aldosterone and cortisol.

Structural regression is also initiated by PGF2a. During structural regression cell mass and total gland size are reduced significantly (corpus luteum diameter < 13 mm; Concepción et al., 2000) as a result of apoptosis. PGF2a initiates both the intrinsic and extrinsic apoptotic signaling pathways (Skarzynski et al., 2008). In extrinsic apoptotic

signaling, receptors on the cell membrane receive signaling from outside the cell to initiate apoptosis. Receptors are commonly TNFR (tumor necrosis factor receptors) or Fas. Both receptors contain a "death domain" inside the cell. Fas ligand (FasL) or TNFalpha bind to their respective receptors. Upon binding, the FADD complex (fas associated death domain) binds to the receptors and initiates a signaling cascade within the cell. FADD activates caspases 8 and 10, the initiator caspases. Initiator caspases signal to effector caspases to induce cellular apoptosis. When an initiator caspase is activated, the pro-domain of the caspase is cleaved rendering it active. A non-cleaved caspase is referred to as a pro-caspase. The initiator caspases 8 and 10 signal for cleaving of the executioner caspases 3, 6 and 7. Caspase 3 is responsible for the cleaving of the inhibitor regulating CAD (caspase activated DNase; Porter et al., 1999). Normally CAD is inhibited to prevent degradation of DNA (Yadav et al., 2005). When caspase 3 cleaves ICAD (Inhibitor of caspase-activated DNase), CAD is active to degrade DNA. Caspase 6 cleaves and degrades lamins (Ruchaud et al., 2002). Lamins are important in nuclear structure and transcriptional regulation (Porter et al., 1999). Caspase 7 inhibits activation of PARP (poly ADP-ribose polymerase). When DNA is damaged, PARP is activated as repair machinery for DNA (Brauns et al., 2005). When PARP is successfully inhibited, DNA is successfully degraded during apoptosis (Elmore et al., 2007).

In intracellular or intrinsic apoptosis, apoptotic signaling is mediated by the mitochondria. Pro-apoptotic factors such as BAX (Bcl-2 associated X protein) and BAD (Bcl-2 associated dead promoter) are translocated to the mitochondria (Elmore et al., 2007). A function of BAX and BAD is to create pores on the outer mitochondrial membrane (Elmore et al., 2007). These pores allow for release of cytochrome c from the

mitochondrial intermembrane space (Elmore et al., 2007). Cytochrome c is an important signaling molecule in intrinsic apoptosis (Elmore et al., 2007). Cytochrome c binds with apaf-1 allowing for recruitment of pro-caspase 9 and formation of the "wheel of death" complex, which subsequently activates caspase 9 (Elmore et al., 2007). Activation of caspase 9 leads to cleaving of pro-caspase 3, 6 and 7 into their active forms. Active caspase 3, 6 and 7 perform the same functions as they do in the extrinsic pathway (Elmore et al., 2007).

PGF2a binds to the FP receptor which is a seven helix G-protein coupled transmembrane receptor (Wiltbank et al., 1995). Upon binding to the receptor, an intracellular signaling cascade is activated, specifically Galpha(q) (Wang et al., 2003). In Galpha(q) signaling, phospholipase C (PLC) is activated (Wang et al., 2003). Activation of PLC leads to the cleavage of phosphatidylinositol 4, 5 bisphosphate (PIP2) and subsequently produces two intracellular signaling molecules, inositol 1, 4, 5 triphosphate (IP3) and diacylglycerol (DAG) (Hou et al., 2008). Both IP3 and DAG perform different functions in the cell. IP3 will translocate to the smooth ER, where it will bind to its respective receptor (Kliem et al., 2007). Upon binding, the ER will release calcium ions (Ca^{2+}). Ca^{2+} inside the cell serves as a secondary messenger. When levels of Ca^{2+} increase intracellularly, they bind to the regulatory subunits of PKC, rendering PKC active. As a kinase, the main function of PKC is to phosphorylate enzymes and proteins that lead to a decrease in progesterone synthesis and trigger apoptosis.

Corpus Luteum Protein Expression

Despite changes in mRNA expression, protein expression must be studied in the corpus luteum in order to adequately analyze abundance of proteins that synthesize

progesterone. Since StAR protein is the rate limiting step in steroid biosynthesis, StAR protein abundance should correlate with progesterone output. StAR mRNA expression appears to decrease during mid luteal cycle and significantly drop during luteal regression. However, StAR protein levels actually appear higher during mid luteal cycle, despite lower expression of mRNA. StAR protein levels also decline during luteal regression (Devoto et al., 2001).

In addition to steroidogenic protein expression, AMPK (AMP-activated protein kinase) is known to regulate expression of key proteins such as CYP11A, StAR, 3B-HSD and HSL (Kohen et al., 2003). Phosphorylation of HSL at Ser-565 prevents activation of HSL by PKA. Inhibiting activation of HSL leads to decreased mobilization of cholesterol from cholesterol-esters, resulting in a decrease in substrate availability for progesterone synthesis.

CYP11A1 mRNA and protein expression also increases in a mature CL, however not as much as StAR protein, given that StAR protein is the rate limiting step of steroid biosynthesis. (Kfir et al., 2018). CYP11A1 protein expression increases during luteal formation. CYP11A1 mRNA decreases rapidly however protein remains relatively unchanged during luteal regression. Despite a 50% reduction in CYP11A1 mRNA at 24 h following PGF2a treatment, CYP11A1 protein remains steady (Atli et al., 2012). Additionally, increase in LH secretion leads to increase in CYP11A1 protein in the corpus luteum. In the rodent model, prolactin signals for increase of CYP11A1 (Taketa et al., 2012). Depletion of either LHCGR or PRLR (prolactin receptor) results in lower amounts of secreted progesterone due to decreased CYP11A1 protein. As pregnenolone

is the first intermediate for all steroid hormones, progesterone cannot sufficiently be synthesized without CYP11A1 protein expression (Rodgers et al., 1995).

3β-Hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3B-HSD) converts pregnenolone into progesterone. This enzyme is expressed in the mitochondrion but in low levels and is greatly expressed in the endoplasmic reticulum. 3B-HSD protein appears in theca cells prior to ovulation. This expression carries into the corpus luteum as theca and granulosa cells are remodeled into small and large luteal cells. During luteal regression, there is a significant decline in 3B-HSD mRNA and protein (Devoto et al., 2001).

Hypoxic Environment of the Corpus Luteum

During PGF2a induced regression, apoptosis occurs which results in a decrease in overall gland size (Skarzynski et al., 2010). One of the main cell types of the corpus luteum are endothelial cells (O'Shea et al., 1989), to support large amounts of vascularization in the gland. As the corpus luteum undergoes structural regression endothelial cells that make up vascular tissue die off, resulting in a hypoxic environment. Blood flow to the corpus luteum is significantly reduced within 8 h following a luteolytic dose of PGF2a (Acosta et al., 2002). This is accompanied by a large reduction in total area of the gland as well as gland mass.

Hypoxia as a result of lack of blood flow has a major effect on progesterone production. A hypoxic environment significantly decreased progesterone production, as compared to luteal cells cultured in a normoxic environment (Hasegawa et al., 2019). mRNA for StAR, CYP11A1 and 3B-HSD decreases in a hypoxic environment; however, there was no significant difference in expression as compared to cells cultured under

normoxic conditions. As oxygen concentration steadily decreases, so does progesterone output (Nishimura et al., 2006). Additionally, as bovine luteal cells are cultured for longer periods of time in hypoxic environments, progesterone output decreases. Upon stimulation with LH, progesterone production increases in a hypoxic environment, however, remains low when compared to progesterone secretion in a normoxic environment when stimulated with LH.

Gene expression appears to also be altered in a low oxygen environment. CYP11A1 mRNA is significantly suppressed during hypoxia (Nishimura et al., 2006). Upregulation of HIF-1 also correlates with a decrease in steroid production. In mouse Leydig cells, testosterone production drops in response HIF-1 activation. Consequentially, StAR mRNA is also decreased during HIF-1 upregulation (Wang et al., 2019).

Mitochondrial Hypoxic Response

The mitochondrion is the main organelle that regulates steroidogenesis. In addition, it is also a major consumer of oxygen required for ATP synthesis. During hypoxia, mitochondrial response is often noted by changes in mitochondrial fission, reactive oxygen species (ROS) production, and regulation of HIF-1 (hypoxia-inducible factor 1; Hamanaka et al., 2009).

The mitochondrion is made up of cristae, folds in the inner membrane allowing the organelle to have increased surface area. Under normal oxygen conditions, mitochondria are long, and can often stretch the length of a cell. This type of morphology favors processes like oxidative phosphorylation and ATP production (Fuhrmann et al., 2017). When oxygen is depleted, mitochondria undergo fission, in which they shorten in

length and divide, leading to smaller segments (Fuhrmann et al., 2017). This can include one large mitochondrion becoming several shorter mitochondria (Fuhrmann et al., 2017). Mitochondrial fission is often a response to low oxygen in an attempt to keep ROS production to a minimum and keep the cell healthier (Chen et al., 2012).

There are a number of ways in which mitochondrial fusion is stimulated. Mitofusin 1 and 2 (Mfn1, Mfn2) are found on the outer membrane of the mitochondrion and are essential for fusion (Plewes et al., 2020). Mfn1 and 2 support mitochondrial fusion in early embryo development, and knockout animals reveal that all embryos are terminated at or before mid-gestation (Chen et al., 2003). This shows that not only is mitochondrial fusion critical for survival, but Mfn1 and Mfn2 are necessary to support early development (Chen et al., 2003). Rate of steroidogenesis is often highly associated with Mfn1 and Mfn2 activation (Duarte et al., 2012). LH is a major up regulator of cAMP, and therefore PKA activation. As cAMP levels increase, mRNA expression for Mfn2 increases (Soodak et al., 1988). This indicates that not only is LH essential for StAR activity and cholesterol shuttling through the mitochondrion but is also involved in processes to regulate mitochondrial fusion. Mfn2 is also hormonally regulated, given that it responds highly to LH. This also shows that mitochondrial fusion is not only hormonally stimulated, but also essential for steroidogenesis. As a result of Mfn2 activation during steroidogenesis, mitochondrial fusion is purely PKA dependent. This is also true for regulation of cholesterol transport. StAR protein is phosphorylated by PKA and MAPK (Duarte et al., 2014). Additionally, increase in Mfn1 and 2 for mitochondrial fusion also occurs, more specifically Mfn2 (Hall et al., 2014). Removing Mfn2 results in decreased steroid production. Adversely, Mfn2 is upregulated both during and after

steroid synthesis, showing a close relationship Mfn 1 and 2 induced mitochondrial fusion and steroidogenesis.

In contrast, dynamin-1-like protein (DRP1) is essential for fission. Upregulation of DRP1 is highly correlated with an increase in fission rates (Fonseca et al., 2019). Additionally, knockout animals for DRP1 are lethal during embryonic development. Proper management of both mitochondrial fission and fusion during early development is critical for survival. Activation of PKA by LH is known to phosphorylate DRP1, therefore rendering it inactive, and preserving the process of mitochondrial elongation (Plewes et al., 2020).

ROS are produced in the electron transport chain (ETC), which occurs on the inner mitochondrial membrane. ROS are most commonly produced at complex III of the ETC, during the formation of ubisemiquinone (Bordt et al., 2017). In a low oxygen environment, HIF1 is activated. However, for HIF1 activation in a hypoxic environment to occur, ROS must be produced for signaling (Hamanaka et al., 2009). ROS production stabilizes HIF1, allowing it to activate. This is generally regarded as a way to activate apoptosis in the cell through endogenous metabolites such as hydrogen peroxide, nitric oxide and superoxides.

In normoxia, HIF1 has the ability to downregulate itself, through the use of dnHIF1a. HIF1 is unable to serve as a transcription factor, when dnHIF1a is present, preventing binding of DNA and gene transcription. In hypoxia, HIF1 can be farther upregulated by Ras/Raf signaling (Bardos et al., 2005). Ras/Raf is activated through a receptor tyrosine kinase (RTK). This in turn activates Raf and MAPK. If MAPK is inhibited, it results in a decrease of HIF1 transcriptional activity. Downstream targets of

the Akt (protein kinase B) signaling pathway are also known to upregulate HIF1, such as mTOR. mTOR is upstream of many metabolic pathways that regulate transcription of genes essential for survival during hypoxia (Land et al., 2007).

Lipid Droplet Hypoxic Response

LDs are intracellular organelles made of cholesterol-ester and triglycerides. The lipid droplet surface consists of a phospholipid monolayer (Guo et al., 2009). In large luteal cells, LDs can be as large as half the cell, or 100 μm in diameter (Olzmann et al., 2019). On the surface, LDs are covered in perilipins. Perilipins are proteins known to protect LDs from lipases found inside the cell, such as HSL. Through PKA activation, perilipases are phosphorylated, which exposes the inner contents of the organelle for HSL lipolysis (Guo et al., 2009).

Despite general LD composition, evidence shows that LDs can be specialized for certain cell types in order to help facilitate a given function. In addition, not all LDs contain the same proteins, and have varying rates of acquiring triglycerides and cholesterol (Olzmann et al., 2019).

Lipid droplet mobilization must take place in order for LDs to be utilized for specific functions, such as steroidogenesis or to provide metabolic energy (Suzuki et al., 2011). This is mediated through G-protein signaling (Suzuki et al., 2001). During activation of the G-protein, adenylyl cyclase is activated, which in turn generates cAMP from ATP. cAMP activates PKA. As stated previously, PKA will phosphorylate HSL as well as perilipin, leading to cholesterol mobilization.

In a hypoxic environment, lipid droplets increase accumulation *in vitro,* suggesting a lipid mediated response to low oxygen (Gordon et al., 1977). Additionally,

this is not directly correlated with HIF1 (Dijk et al., 2017). As luteal cells become increasingly more hypoxic, lipid droplets inside these cells are forced to adapt to this environment.

Omega-3 Fatty Acids

Literature has shown that polyunsaturated fatty acids (PUFA) improve reproductive performance in cattle (Burns et al., 2003; Burke et al., 1997). The 3 most common omega-3 fatty acids are alpha linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6; Harnack et al., 2009). Both EPA and DHA are common long-chain fatty acids found in fish oil. Recent data has shown that supplementation with fish oil in bovine luteal cells *in vitro* leads to increased membrane fluidity (Plewes et al., 2017). In addition, single particle tracking of the PGF2a FP receptor showed an increase in lateral and spatial mobility most likely due to increased membrane fluidity (Plewes et al., 2017; Plewes et al., 2018). There is also an increase in plasma membrane lipid composition of EPA and DHA in luteal cells following fish oil supplementation. Additionally, cows supplemented with either fish oil or meal have increased EPA and DHA in blood plasma (Burns et al., 2003; Wamsley et al., 2005; White et al., 2012; Plewes et al., 2017; Plewes et al., 2018), luteal tissue (White et al., 2012; Plewes et al., 2017) and uterine endometrium (Burns et al., 2003).

Dietary supplementation of fish meal reduces luteal sensitivity to intrauterine infusion of PGF2a (Plewes et al., 2018). Surprisingly, steady-state mRNA that regulate progesterone synthesis were reduced and did not differ from cows with a regressed corpus luteum. There are two possibilities for this reduction in steady-state mRNA despite elevated progesterone. In the study of Plewes et al. (2018), luteal tissue was

collected during the first 18 h following PGF2a infusion. It is highly possible that tissue sampling frequency was inadequate to detect a potential rebound in steady-state mRNA following PGF2a. A second possibility is that fish oil or meal supplementation could protect mitochondria during PGF2a induced hypoxia allowing for maintenance of progesterone synthesis.

Specific Aims

This study examined the effects of PGF2a, fish oil and hypoxia on corpus luteum function, progesterone synthesis, mitochondrial morphology and lipid droplet dynamics in the bovine corpus luteum, both *in vitro* and *in vivo.*

The long-term goals of this study were to decrease luteal sensitivity to PGF2a through omega-3 fatty acid supplementation, as well as understand the effects of low oxygen on steroidogenesis.

Aims, Research Questions and Hypotheses

A1 Determine if steroidogenic steady state mRNA rebounds following PGF2a intrauterine infusion in fish oil supplemented animals.

Q1 What are the effects of omega-3 fatty acid treatment on steroidogenic enzyme mRNA levels over 48 h of PGF2a treatment?

H1 Cows supplemented with fish oil will retain steroidogenic enzyme mRNA following PGF2a treatment out to 48 h.

A2 Determine correlation between steroidogenic gene mRNA and steroidogenic protein abundance following intrauterine infusion of PGF2a.

Q1 What are the effects of omega-3 fatty acid treatment on steroidogenic protein levels over 48 h of PGF2a treatment?

H2 Cows supplemented with fish oil will retain steroidogenic protein levels in luteal tissue following PGF2a treatment for 48 h.

A3 Omega-3 fatty acids are incorporated into the mitochondrial membrane, making it resilient to hypoxic environments, and able to synthesize adequate progesterone.

Q1 What are the effects of hypoxia on bovine luteal cell progesterone output when treated with fish oil?

H3 Bovine luteal cell progesterone synthesis will be unaffected by hypoxia when prior treated with fish oil.

Q2 What are the effects of hypoxia on bovine luteal cell mitochondrial morphology when treated with fish oil?

H4 Bovine luteal cell mitochondria will show greater amounts of fusion in a hypoxic environment when supplemented with fish oil.

A4 Effects of fish oil on luteal cell lipid droplets accumulation in whole animal luteal tissue biopsies.

Q1 What are the effects of fish oil on lipid droplet size and accumulation in the bovine corpus luteum?

H5 Cows supplemented with fish oil will show an increase in lipid droplet size and accumulation when compared to those supplemented with vegetable oil.

Conclusion

Early bovine embryo death is a leading cause of infertility in the United States beef and dairy industry. There are a large number of causes responsible for early embryo termination in the bovine. One leading cause is a lack of established communication between the embryo and the maternal environment. This can lead to regression of the corpus luteum and the embryo is terminated. This presents a critical problem in reproductive biology

This study was designed to determine the effects of PGF2a treatment on bovine luteal mRNA and protein responsible for steroid production in response to fish oil treatment. Additionally, the effects of low oxygen on mitochondrial dynamics and

progesterone synthesis were observed in response to fish oil treatment. The results of this study present a cost-effective method for maintaining early bovine embryo development and therefore, a potential solution for the United States beef and dairy industry.

CHAPTER II

METHODOLOGY

Animal Husbandry

Cows were purchased from a local sale barn and housed at the Animal Reproduction Biotechnology Laboratory at Colorado State University in Fort Collins, CO. All animal experimental protocols were approved by the Colorado State University institutional animal care and use committee (Approval #16-6761AA). An Aloka 500 V ultrasound machine with a 5 MHz linear array transducer was used to scan ovaries for abnormalities (cystic follicle) and uteri for pregnancies. Cows were removed from the study for presence of abnormal ovaries or pregnant. Body weights were collected, and cows were stratified by body weight and randomly divided into 2 dietary supplementation groups – vegetable oil (n = 10) and fish oil (n = 11). Diets consisted of 95% mixed grass hay and 5% supplement that were isocaloric and isonitrogenous (Tables 1 and 2) and met or exceeded NRC recommendations for non-lactating cows. Cows were individually penned from 0600 to 0900 h and fed supplements and hay, after which cows were turned out of pens to have access to water and shelter. Cows were penned in the evening (1600 to 1800 h) to consume remaining hay. Diets were fed at 2% dry matter intake approximately 70 days. Cows were administered an intramuscular injection of 25 mg of PGF2a on day 50 of supplementation to synchronize estrous cycles. Cows were observed for estrous behavior at 0500 and 2100 h for a minimum of 30 min. Estrotect patches were

applied to tail-head region of the animal to aid in detection of estrus. Cows not detected in estrus within 5 days were administered a second injection of PGF2a and were monitored for an additional 5 days for estrous behavior.

Jugular blood samples were taken at days 0, 35 and 70 to monitor changes in plasma fatty acid composition. Samples were centrifuged at 1500 x g for 15 min at 4 °C and plasma was stored at -80 °C until gas chromatography (GC) analysis.

Luteal Tissue and Blood Collection

Luteal biopsies and blood were collected over a period of 48 h on day 10 to 12 following synchronized estrus. Intrauterine infusions of either 0.25 mL saline or 0.5 mg PGF_{2a} were administered at h 0 and 12 in the uterine horn ipsilateral to the ovary containing the CL. Blood samples were taken from the jugular vein every 3 h for 24 h of experimental period, and every 6 h for remaining 24 h of experimental period and assayed for serum progesterone. Blood samples were allowed to clot and kept at 4 °C prior to centrifugation at 1500 x g for 10 min. Luteal biopsies were taken at h 0, 18, 24, 36 and 48. Prior to biopsy, the vulva was cleaned with betadine surgical scrub, and a local block of 2% lidocaine (3-6 mL in the tail head) was administered. A transvaginal ultrasound guided biopsy probe equipped with a spring loaded Quick-Core 60 cm 18-guage with a 20 mm specimen notch needle (QC-18_60-20T, Cook Medical) was used for luteal biopsy collection. The biopsy probe was inserted into the vagina and the ovary containing the corpus luteum was aligned with the projected needle line trajectory (Plewes et al., 2018). The triggering device was activated to collect luteal tissue. Tissue was removed from the biopsy notch (approximately 5 mg), rinsed with sterile 1x PBS and

placed in a sterile 1.7 mL tube for freezing. Tubes were frozen in liquid nitrogen and stored at -80 °C until RNA preparation.

Ribonucleic Acid Extraction and Quantitative Polymerase Chain Reaction

RNA was extracted using the TRIzol plus RNA kit according to the protocol. A 2.0 Qubit Fluorometer was used to estimate RNA concentration and samples were kept at -80 °C until further processing. cDNA was prepared from 1 μg of total RNA using a Bio-Rad T100 PCR Thermal Cycler. Bovine specific primers for genes of interest have been validated and were used in the current study. Primers were designed from Primer3 software from the NCBI gene database. qPCR reactions were carried out in duplicate using a Bio-Rad CFX384 real-time PCR system. Melt curve analysis was conducted to determine single product amplification.

Immunohistochemistry

Luteal tissue biopsies were removed from the biopsy notch, rinsed with sterile 1x PBS and placed in 500 μL of 4% paraformaldehyde (wt:vol) for 1 h at 4 °C. Luteal samples were immediately rinsed with 1x PBS and placed in 1 mL 20% sucrose (wt:vol) solution and incubated for 24 h at 4 °C. Following sucrose dehydration, biopsy samples were placed in a 10 x 10 x 5 mm biopsy tissue mold (VWR 25608-922) containing Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura, 4583). The molds were submerged in 50 mL of isopentane and placed in Styrofoam containing liquid nitrogen. Following freezing, biopsies were wrapped in parafilm and aluminum foil and stored at -80 °C. Frozen biopsies were sliced at 15 μm sections using a Leica cryostat (CM1950; -20 °C) and placed onto glass microscope slides (VWR 48300-026). Microscope slides were stored at -80 °C until further processing.

Slides were warmed to room temperature and tissue slices were washed 3 times with 1x PBS to remove excess OCT compound. Sections were then permeabilized using 1.0% Triton-X 100 (Sigma 9002-93-1) for 30 min. After permeabilization, tissue samples were washed 3 times with 1x PBS. A blocking buffer (1% BSA and 5% normal goat serum; Thermo 50062Z; Sigma A8806) was applied to the slides and incubated for 30 min. Slides were then washed with 1x PBS and the primary antibody (CYP11A1) was applied at the appropriate dilution as shown in Table 3. The slides were incubated with the primary antibody overnight at 4 °C. After incubation with primary antibody, the appropriate secondary antibody was applied to the slides at the correct dilution. Hoechst 33342 was used to counterstain cell nuclei.

Images were taken on a Zeiss LMS 700 confocal with the appropriate filters. Tissue sections were observed using a 20x objective (N.A. = 0.8) and a 100x oil immersion objective (N.A. = 1.4). Images were captured using Zen Black 2011 software. Images were analyzed for protein amount using mean gray area analysis in ImageJ.

Slides containing tissue samples were warmed to room temperature prior to staining. Tissue was washed 3 times with 1x PBS prior to staining and subsequently stained with 10 μM BODIPY 493/503 for 30 min to stain lipid droplets. Slides were then washed and stained with 2.5 μg/mL of Hoechst 33342 and Phalloidin 633 at a 1:33 dilution. Hoechst and Phalloidin were incubated for 15 min on the samples diluted in 1x PBS. Hoechst and Phalloidin were washed off of slides and slides were analyzed using confocal microscopy. A DAPI filter was used for Hoechst 33342, FITC filter for BODIPY 493/503, and TRITC for AlexaFluor633.

Corpus Luteum Collection, Digestion and Culture

Bovine ovaries were collected at a local slaughterhouse in Greeley, CO, and transported to the laboratory at the University of Northern Colorado in 1x PBS. The ovaries were then submerged in 70% ethanol to sterilize the ovary. The corpus luteum was dissected from the ovary and the capsular connective tissue was removed from the gland. The corpus luteum was sliced into 500 μm sections using a handheld microtome and enzymatically digested with collagenase I (2000 units/mg). Harvested cells were grown in T-75 or T-25 cell culture flasks containing Ham's F12 with 5% fetal bovine serum, insulin (5ug/mL), transferrin (5ug/mL), selenium (5ng/mL), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/mL amphotericin B (pH 7.34). Cells were grown to a monolayer until ready for experimentation. Only healthy adhered cells were used for experimentation based on confluency. Cells were incubated at 37 °C and in a humidified atmosphere of 95% air and 5% CO_2.

Fish Oil Treatment

For all experiments, lipids from fish oil were bound to BSA prior to addition in culture medium. In brief, 0.03% fish oil (v:v) was added to Ham's F12 culture medium containing 33 mg/mL of fatty acid free BSA. BSA control medium was also prepared using 33 mg/mL of fatty acid free BSA. Media were incubated in a shaking water bath at 37 °C for 2 h prior to experimentation to allow binding of lipids in fish oil to BSA. Cells were cultured in BSA or fish oil treated media for 72 h at 37 °C in a 95% humidified air atmosphere and 5% CO_2 to allow for incorporation of long-chain fatty acids into biological membranes.

Oxygen Treatment

Cells were incubated in either a normoxic (20% O_2) or hypoxic (5% O_2) environment for 24 h. A hypoxic environment was created using a hypoxia incubator chamber (Stem Cell Technologies). Dishes or flasks of cells were placed in the chamber and sealed. A gas line was connected to the inlet valve of the chamber and was purged with a 5% O_2 90% N_2 and 5% CO_2 gas mixture for 2 min. The inlet and outlet valves were then clamped. Normoxic cells were cultured in normal incubator conditions. Both normoxic cells and hypoxic cells (in chamber) were placed in an incubator at 37 °C and in an atmosphere of 95% air and 5% CO_2.

Effects of Hypoxia on Fish Oil Treated Luteal Cell Progesterone

Six 24 well dishes (5×10^4 cells/well) were prepared from 3 corpora lutea for use in progesterone analysis. Cells were treated with either 0.03% fish oil or BSA control for 72 h. After treatment, cells were stimulated with either 10 μM of forskolin in a final volume of 1 mL. All treatments were applied in triplicate. During collection, 50 μL of media was collected at 0, 6 and 24 h from each well and combined into a total of 150 μL for each treatment. Spent culture media was assayed for progesterone using an ELISA (Cayman chemical 582601) per manufacture's protocol

DNA was extracted from cells within each well using TRIzol reagent to normalize cell number. In brief, 100 μL of TRIzol was added to each well. The wells were scraped, and the cell lysate was collected. DNA was extracted using the TRIzol reagent protocol from Invitrogen and quantified using a 2.0 Qubit Fluorometer. In brief, luteal tissue was homogenized on ice in 100 μL of TRIzol reagent. An additional 900 μL of TRIzol was

added and allowed to incubate for 5 min at room temperature before adding 200 μL of chloroform. Tissue samples were vortexed and incubated for an additional 3 min. Samples were then centrifuged at 12,000 x g for 15 min at 4 °C. The aqueous phase was collected and transferred to a new 1.7 mL tube. An equal volume of 70% ethanol was added to the sample and vortexed. Samples were added to spin columns and centrifuged at 12,000 x g for 15 seconds. 700 μL of wash buffer I was added to each spin column and centrifuged at 12,000 x g for 15 seconds and this step was repeated. 500 μL of wash buffer II was used to wash each sample. Columns were then centrifuged at 12,000 x g for 15 seconds at room temperature. DNA was eluted into collection tubes with 30 μL of nuclease free water.

Data Analysis

Effects of hypoxia and fish oil on bovine luteal cell progesterone synthesis were analyzed using three-way analysis of variance. The statistical model included oxygen environment (normoxic or hypoxic), treatment (BSA-control or fish oil), time and all possible interactions as sources of variation.

Effects of Hypoxia on Fish Oil Treated Luteal Cell Mitochondria

Cells from 7 to 8 corpora lutea were used in this experiment. Cells were cultured in 35mm confocal microscopy dishes with a cover slip bottom (5×10^4 cells/dish). Cells were grown to confluency and then randomly treated with either fish oil or BSA supplemented medium. Cells were incubated in treatments for 72 h at 37 °C. Cells were then incubated in normoxic or hypoxic environments for an additional 24 h (either 20% or 5% O_2).

After 24 h of incubation, cells were stained by directly adding 100 nM tetramethylrhodamine, methyl ester, perchlorate (TMRM) to each dish. An additional set of dishes were treated with Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (CCCP; 1 ng/mL) for 30 min to serve as a positive control. Unstained cells were used as a negative control. Cell nuclei were counterstained using Hoechst 33342 (2.5 μg/mL).

ImageJ Analysis

All confocal data were analyzed using ImageJ (FIJI; Version 2.0.0-rc-43/1.52g). Mitochondrial analysis was performed using protocols previously established by Valente et al. (2017). In brief, mitochondria were assessed for membrane potential, mitochondrial fragment size, mitochondrial fragment number, mitochondrial footprint, mitochondrial mean branch number and mitochondrial mean junction number. Mitochondrial contrast was enhanced, and tubeness was applied to separate individual organelles. Images were then binarized or skeletonized to remove additional material and particle analysis was run. Lipid droplet analysis was performed using ImageJ particle analysis plugin.

Data Analysis

Effects of hypoxia on membrane potential, mitochondrial fragment size, mitochondrial fragment number, mitochondrial footprint, mitochondrial mean branch number and mitochondrial mean junction number were analyzed using three-way analysis of variance. The statistical model included oxygen environment (20 vs 5%), treated (fish oil or BSA) time (0 or 24 h) and the interaction as sources of variation in the statistical model.

Statistical Analysis

All statistical calculations were performed using mixed linear model procedures of SAS. When main effects or interactions were significant ($p < 0.05$) individual means were compared using preplanned pairwise *t*-test.

Table 1. Ingredient, chemical composition, and long-chain fatty acid profile of dietary supplementation

	Experimental Diet	
Item	Vegetable oil	Fish oil
Dry Matter Intake, %	5	5
Ingredient of Supplement, %		
Corn Gluten meal	5	5
Chemical Analysis		
Dry Matter, %	94.1	94.0
Crude Protein, %	39.8	40.1
Degradable Intake Protein, %	29.5	29.9
Undegradable Intake Protein, %	33.7	34.2
Total Digestible Nutrients, %	68.8	69.6
Crude Fat, %	8.4	8.2
Fatty Acid Composition of Supplement, wt, %		
Palmitic Acid (16:0)	15.1	26.7
Palmitoleic Acid (16:1)	<0.5	10.7
Stearic Acid (18:0)	18.2	8.1
Oleic Acid (18:1)	1.5	9.1
Linoleic Acid (18:2)	43.1	1.4
Alpha-Linolenic Acid (18:3)	<0.5	1.4
Arachidonic Acid (20:4)	<0.5	1.1
Eicosapentaenoic Acid (20:5)	<0.5	6.0
Docosahexaenoic Acid (22:6)	<0.5	5.9

Table 2. Chemical composition of Alfalfa Orchard mixed grass hay

Dry matter intake, %	95
Chemical Analysis	
Dry Matter	91
Water Soluble Carbohydrates, %	8.0
Neutral Detergent Fiber, %	47.3
Acid Detergent Fiber, %	34.7
Simple Sugars, %	5.3
Starch, %	1.3
Non Fiber Carbohydrates, %	1.0
Crude Protein, %	19.7
Crude Fat, %	2.7

Table 3: Antibodies used for microscopy

Antibody name	Dilution ratio	Species specificity	Source	Supplier (distributor, town, country)	Cat. No
STAR	1:20	Mouse	Rabbit pAB	Abcam	ab96637
CYP11A1	1:50	Mouse	Rabbit mAB	Cell Signaling	14217
BODIPY 493/503	10 - 20 µM	All		Thermo Fisher (Carlsbad, CA, USA)	D3922
Hoechst	20 nM	All		Thermo Fisher	62249
Phalloidin	1:33	All		Thermo Fisher	A34055
Alexa Fluor 555	1:500	All			
Alexa Fluor 568	1:500	All			
TMRM	1:1000	All			

steroidogenic acute regulatory protein (STAR); Cholesterol side-chain cleavage enzyme (CYP11A1); tetramethylrhodamine (TMRM)

CHAPTER III

RESULTS

Changes in Plasma Fatty Acid Composition

During the supplementation period, blood was drawn at days 0, 35 and 70 to measure plasma long-chain fatty acid composition. Figure 4 shows changes in blood plasma compositions of omega-6 fatty acids in the form of linoleic acid (18:2) and arachidonic acid (20:4). No significant differences were observed following supplementation in omega-6 fatty acids. No significant changes were seen in alpha-linolenic acid (18:3) during the course of the supplementation period. Eicosapentaenoic acid (20:5) was significantly increased in fish oil treated animals at day 70 ($p < 0.05$). Docosahexaenoic acid (22:6) was significantly increased in fish oil animals at both days 35 ($p < 0.01$) and 70 ($p < 0.001$).

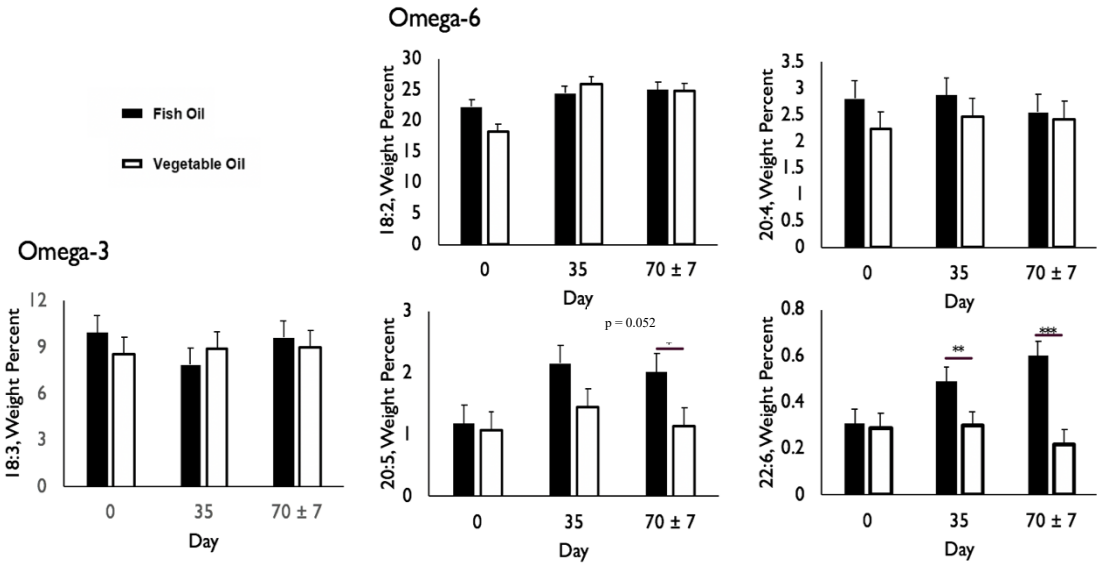

Figure 4: Effects of fish oil and vegetable oil on plasma omega-3 and omega-6 fatty acids in the blood plasma of the bovine. Cows supplemented with fish oil (n = 11) or vegetable oil (n = 10) for approximately. 70 days. At days 0, 35 and 70, blood was drawn to assess plasma long chain fatty acid composition. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Serum Progesterone and Corpus Luteum Diameter

Serum progesterone was measured immediately prior to treatment (0 h), every 3 h for the first 24 h and every 6 h for the remaining 24 h following PGF2a or saline infusion. Corpus luteum diameter was measured using ultrasonography at 0, 18, 24, 36 and 48 h. Animals were sorted into 4 groups based on serum progesterone concentration at 48 h following PGF2a treatment – functional corpora lutea serum progesterone > 1 ng/mL and regressed serum progesterone < 1 ng/mL: fish oil regressed (FOR; n = 2), fish oil non regressed (FONR; n= 6), vegetable oil regressed (VOR; n= 3) and vegetable oil non regressed (VONR; n = 4). In saline treated animals, there was no difference between fish oil and vegetable oil supplementation on corpus luteum diameter (Figure 5A). Overall, corpus luteum diameter was significantly larger for FONR animals, compared to FOR

and VOR ($p < 0.001$). Additionally, VONR had a significantly larger corpus luteum diameter than both FOR and VOR animals ($p < 0.01$) following PGF2a treatment.

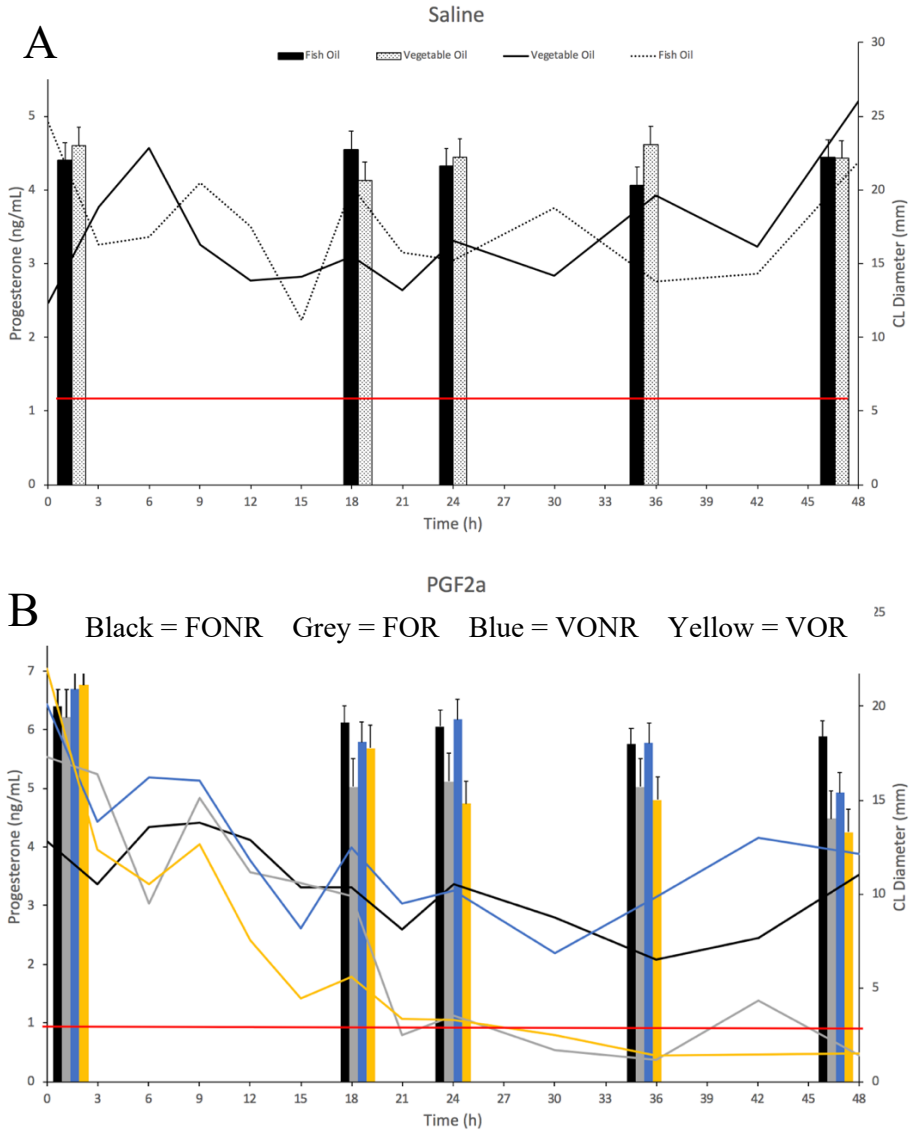

Figure 5: Effects of saline or PGF2a on serum progesterone and corpus luteum diameter in fish oil and vegetable oil supplemented cows. Saline (panel A): Black bar and solid line indicates fish oil supplemented animals (n = 3), grey bar and dashed line indicates vegetable oil supplemented animals (n =3). PGF2a (panel B): Black = FONR (n = 6), Grey = FOR (n =2), Blue = VONR (n = 4), Yellow = VOR (n = 3). Double y-axis graph indicates serum progesterone on primary y-axis as a line graph (ng/mL) and corpus luteum diameter on secondary y-axis as a bar chart (mm). Red line on both graphs indicates functional corpus luteum regression (progesterone < 1 ng/mL).

Steroidogenic Gene Expression

Luteal biopsies taken at 0, 18, 24, 36 and 48 h and were used to assess steady-state mRNA levels for key genes that regulate progesterone synthesis. StAR, CYP11A1 and LDLR steady-state mRNA was significantly higher for animals infused with saline, when compared to PGF2a infused animals (Figure 6A, B, C). Regardless of corpus luteum function, animals infused with PGF2a had a significant reduction in mRNA for all genes measured (Figure 6A, B, C). However, at 48 h, steady-state mRNA for CYP11A1 and LDLR in luteal tissue collected from fish oil supplemented cows was higher as compared to tissue collected from vegetable oil supplemented cows treated with PGF2a ($p = 0.01$; Figure 6A, B, C). There was a tendency for StAR mRNA to be higher in tissue collected from cows supplemented with fish oil ($p = 0.10$; Figure 6A, B, C).

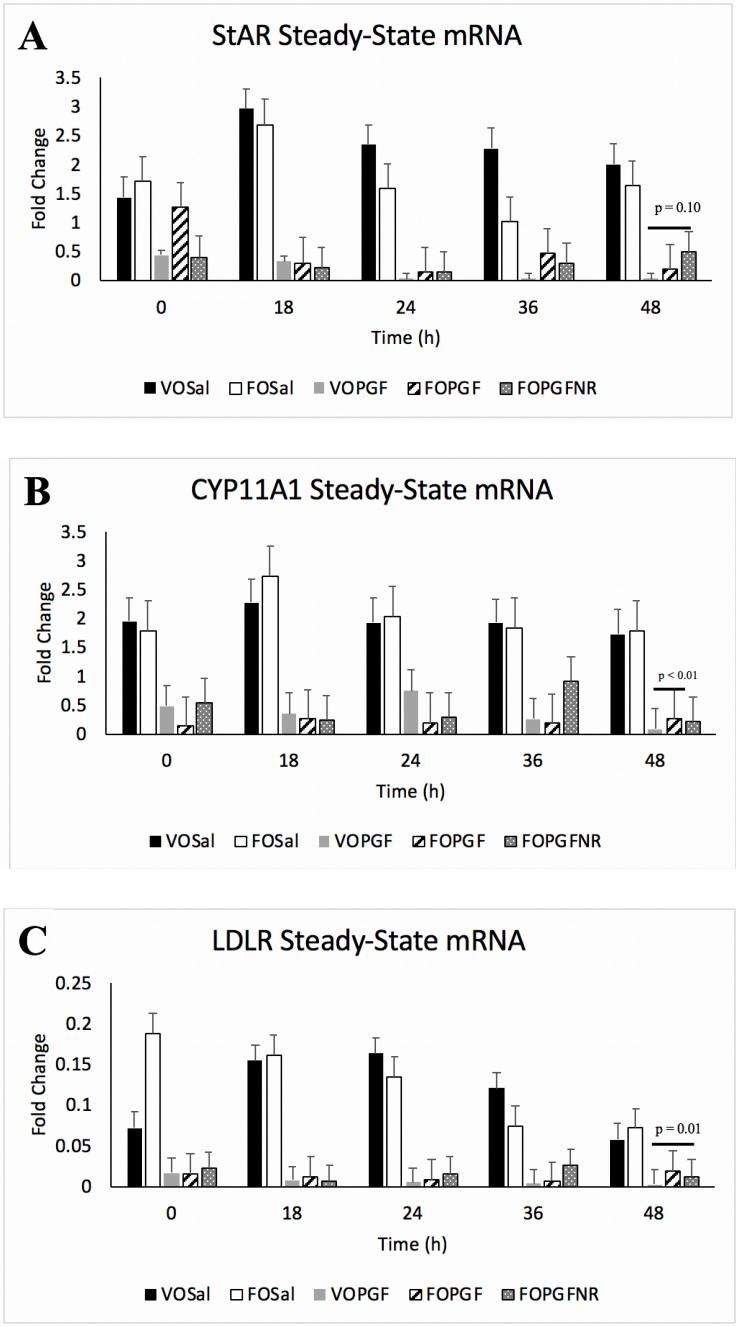

Figure 6: Effects of fish oil supplementation and PGF2a infusion on StAR, CYP11A1 and LDLR steady-state mRNA. Biopsy tissue was collected and assessed for StAR (A), CYP11A1 (B) and LDLR (C) steady-state mRNA using qPCR. Animals received either saline or PGF2a infusions at 0 and 12 h. Tissue was collected at 0, 18, 24, 36 and 48 h. Black bar indicates vegetable oil saline animals (VOSal; n = 3), white bar indicates fish oil saline animals (FOSal; n = 3), grey bar indicates vegetable oil PGF2a animals (VOPGF; n = 7), diagonal bar indicates fish oil PGF2a animals (FOPGF; n = 6) and dotted bar indicates fish oil PGF2a animals that did not regress (FOPGFNR; n = 2).

Steroidogenic Protein Abundance

Biopsies were used to assess protein abundance in luteal tissue. Protein expression was measured using immunohistochemistry and ImageJ analysis. Protein abundance was assessed at 0, 18 and 48 h for CYP11A1. Protein abundance remained unchanged in animals treated with saline, and for cows with a functional corpus luteum (Figure 7). However, protein abundance tended to decrease in response to PGF2a for vegetable oil supplemented cows that had a regressed corpus luteum ($p = 0.13$).

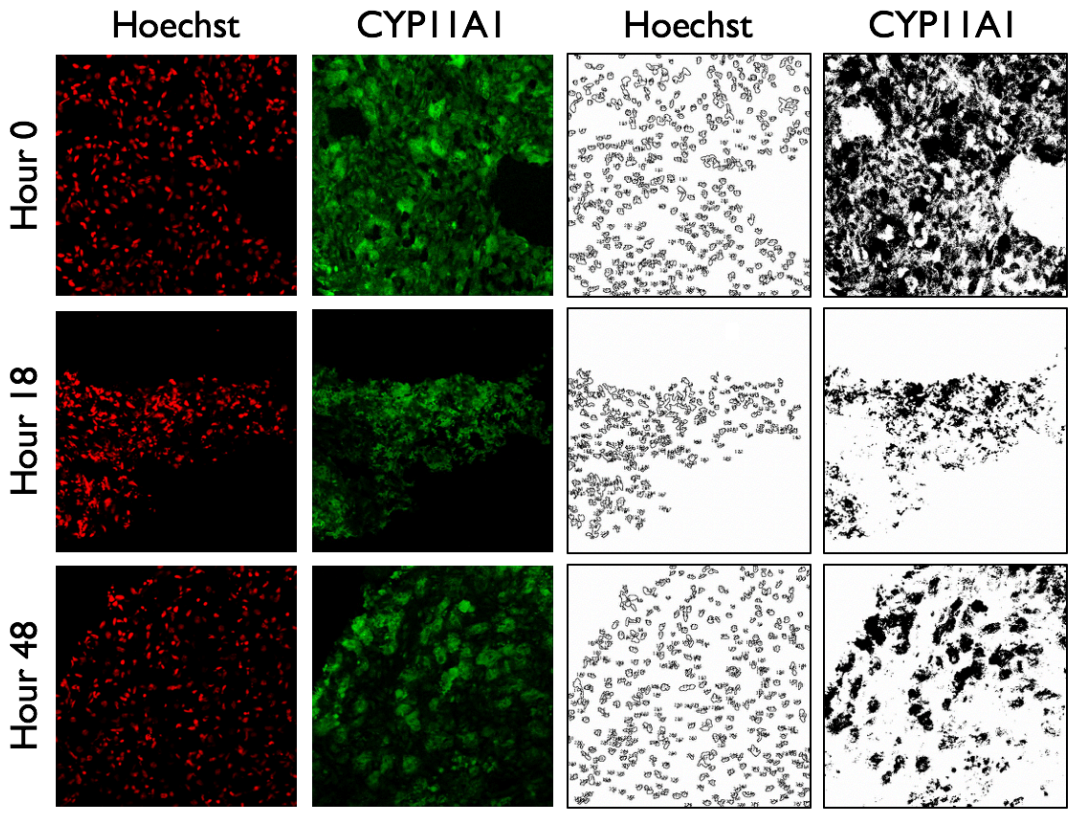

Figure 7: Effects of fish oil supplementation and PGF2a on steroidogenic protein abundance. Representative image at 0, 18 and 48 h showing CYP11A1 abundance. Nuclei staining in red (Hoechst 33342) and CYP11A1 protein abundance (green).

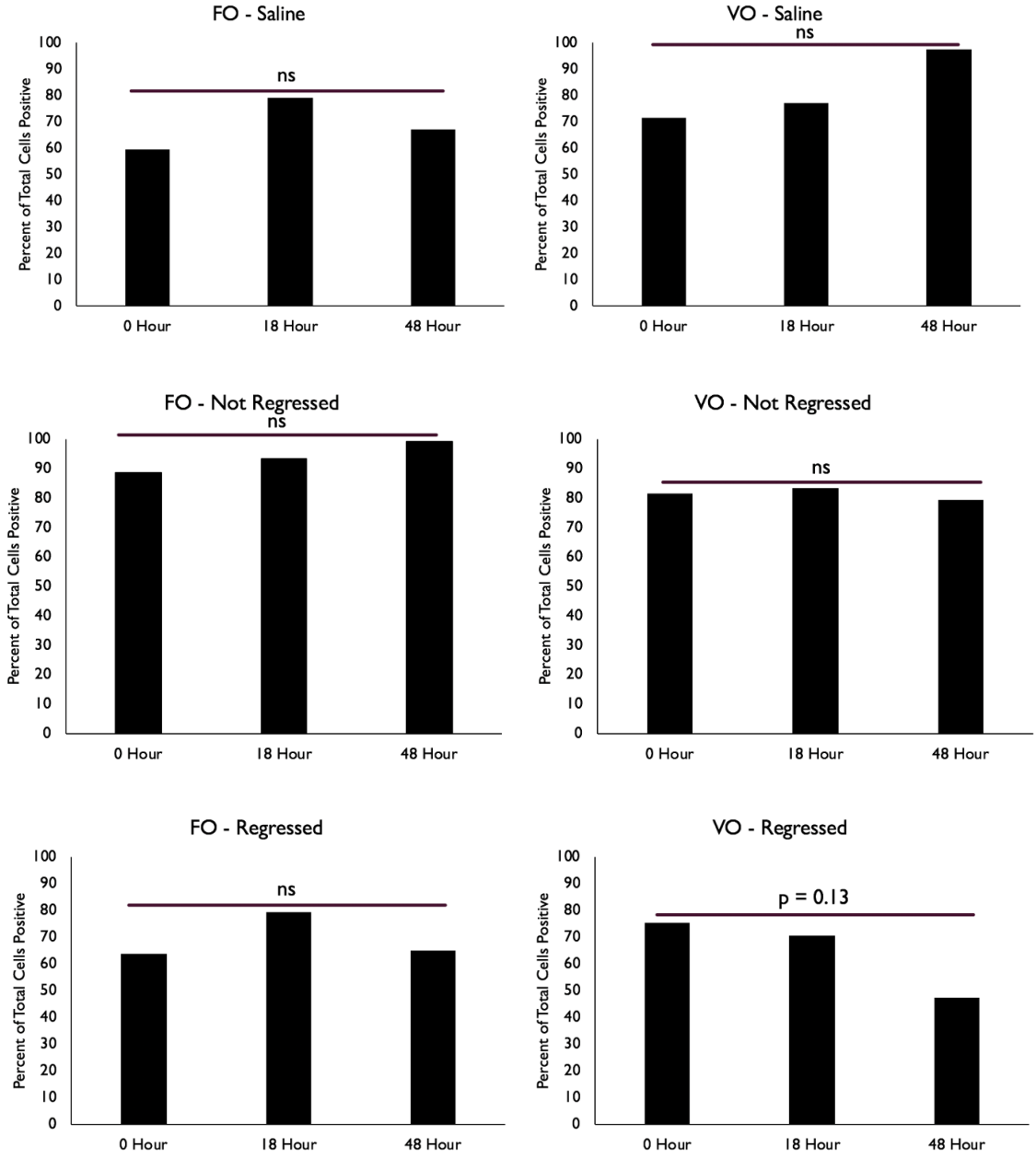

Figure 8: CYP11A1 protein abundance quantification for saline, not regressed and regressed corpora lutea. Quantification was measured as percent of protein positive cells compared to total cells. Top panel shows saline control, mid panel shows non-regressed luteal tissue and bottom panel shows regressed luteal tissue. Fish oil saline n = 3, vegetable saline n =2, fish oil not regressed n = 2, vegetable oil not regressed n = 3, fish oil regressed n = 2, vegetable oil regressed n = 2.

Changes in Lipid Droplet Dynamics

Luteal biopsies at 0 h were used to measure lipid droplet number and size. Effects of fish oil on cultured bovine luteal cell lipid droplets were also measured *in vitro*. Lipid droplet number and size were increased (Figure 9B, C) in fish oil supplemented animals as compared to vegetable oil supplemented animals ($p < 0.01$). In addition, lipid droplet number was increased in fish oil treated bovine luteal cells *in vitro* ($p < 0.01$). However, lipid droplet size was decreased (Figure 9E, F) in fish oil-treated bovine luteal cells *in vitro* as compared to BSA-control bovine luteal cells ($p < 0.01$).

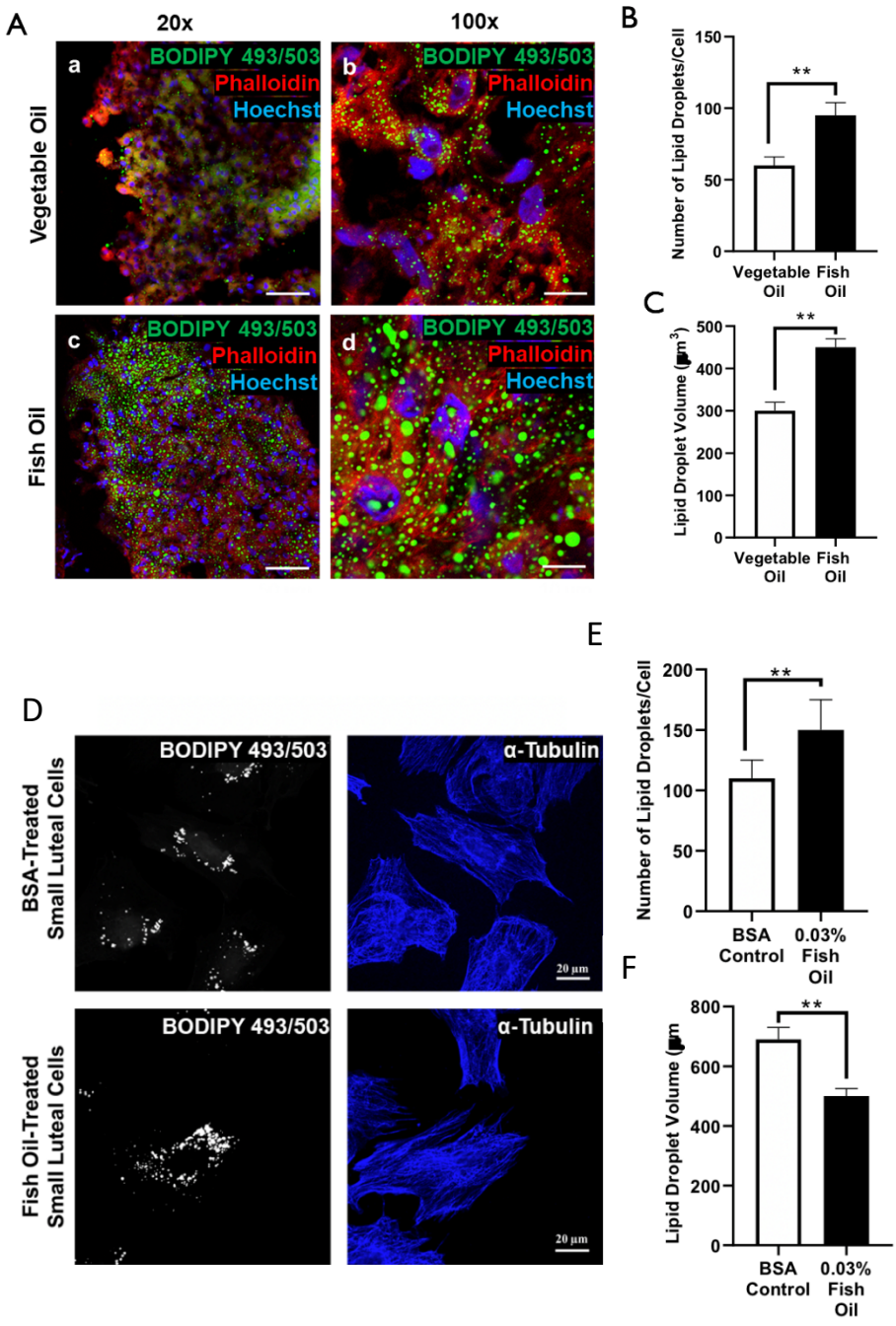

Figure 9: Effects of fish oil on bovine luteal cell lipid droplet accumulation and volume. Luteal tissue was collected following approximately 75 days of supplementation with fish (n = 5) or vegetable oil (n = 4). Cultured cells were treated with fish oil or BSA for 72 h. A) Representative images showing lipid droplet staining in bovine luteal tissue. B) Lipid droplet accumulation in bovine luteal tissue. C) Lipid droplet volume in bovine luteal tissue. D) Representative images showing lipid droplet staining in cultured bovine luteal cells. E) Lipid droplet accumulation in bovine luteal cells *in vitro.* F) Lipid droplet volume in bovine luteal cells *in vitro.* (** $p < 0.01$)

Effects of Fish Oil and Hypoxia on Progesterone Biosynthesis

Progesterone production was measured from cultured luteal cells during hypoxia and treated with fish oil. Forskolin was added to cells to stimulate progesterone synthesis. Progesterone was measured at 0, 6 and 24 h of hypoxia in fish oil treated cell. After 24 h, luteal cells cultured in a hypoxic environment produced less progesterone as compared to luteal cells cultured in a normoxic environment (Figure 10). Additionally, there was no difference in progesterone production between cells treated with fish oil or BSA that were cultured in either normoxic or hypoxic environments (Figure 10).

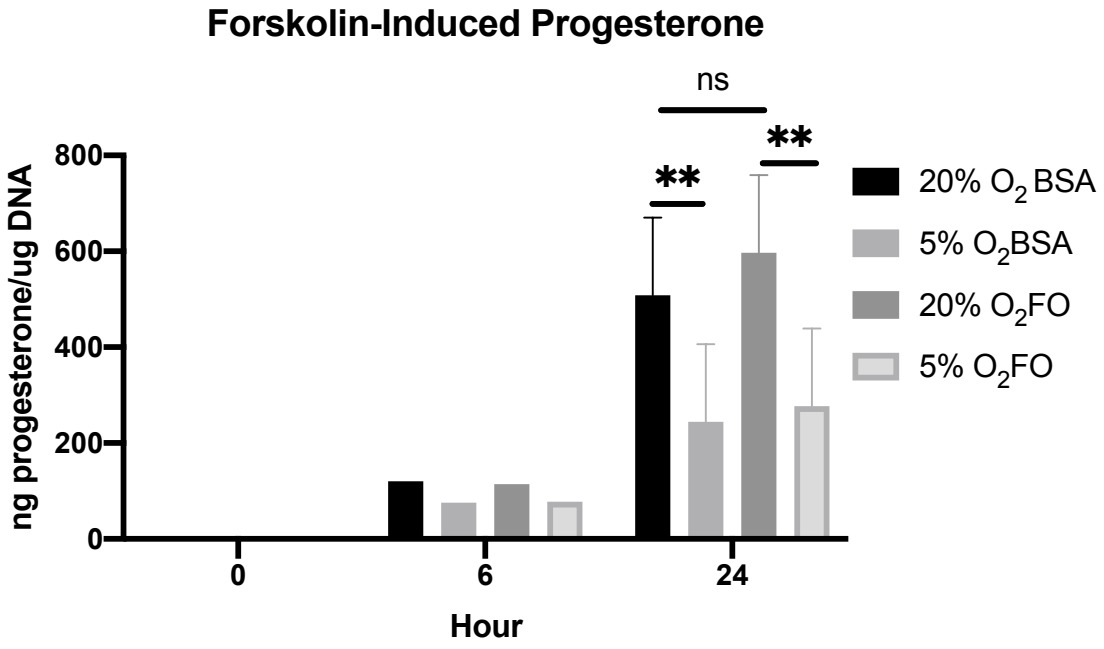

Figure 10: Effects of fish oil and hypoxia on forskolin-induced (10 μM) progesterone production. Cultured bovine luteal cells were treated with fish oil or BSA control for 72 hours and cultured in either 20 or 5% O_2 environments. Progesterone was measured at 0, 6 and 24 h of oxygen incubation. BSA = bovine serum albumin control, FO = 0.03% fish oil (** $p < 0.01$, ns = not significant)

Effects of Fish Oil and Hypoxia on Bovine Luteal Cell Mitochondrial Membrane Potential and Morphology

Mitochondrial membrane potential was assessed using TMRM fluorescence intensity. There was no difference in membrane potential between cells cultured in a normoxic or hypoxic environment. Furthermore, fish oil treatment had no effect on mitochondrial membrane potential.

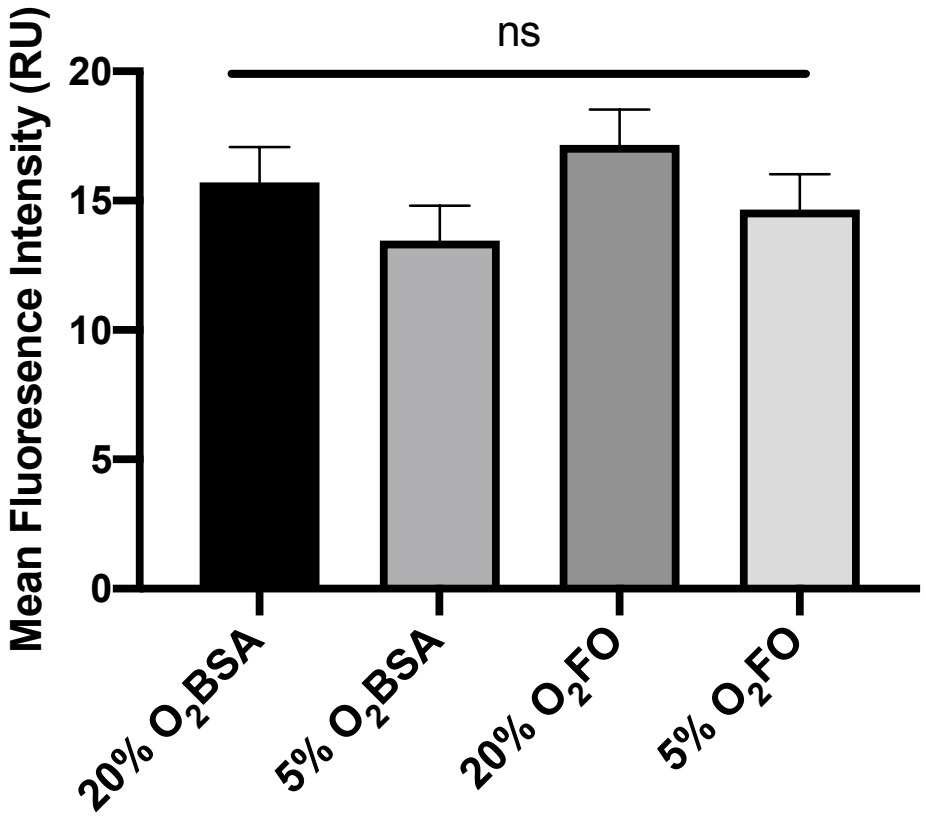

Figure 11: Effects of fish oil and hypoxia on mitochondrial membrane potential. Mitochondrial membrane potential was measured using TMRM following treatment with either BSA supplemented medium or fish oil supplemented medium, and hypoxic or normoxic culture. BSA = bovine serum albumin control, FO = 0.03% fish oil (ns = not significant)

Additionally, the effects of hypoxia and fish oil on mitochondrial fragment size and number were determined. There was an increase in mitochondrial fragment size in luteal cells cultured in a normoxic environment as opposed to a hypoxic environment,

regardless of fish oil or BSA supplemented medium ($p < 0.05$; Figure 12B). Furthermore, fish oil treatment mitigated this decrease in fragment size when cultured in a hypoxic environment (Figure 12B). Mitochondrial fragment size was reduced in luteal cells treated with CCCP regardless of oxygen environment or supplementation (Figure 12C).

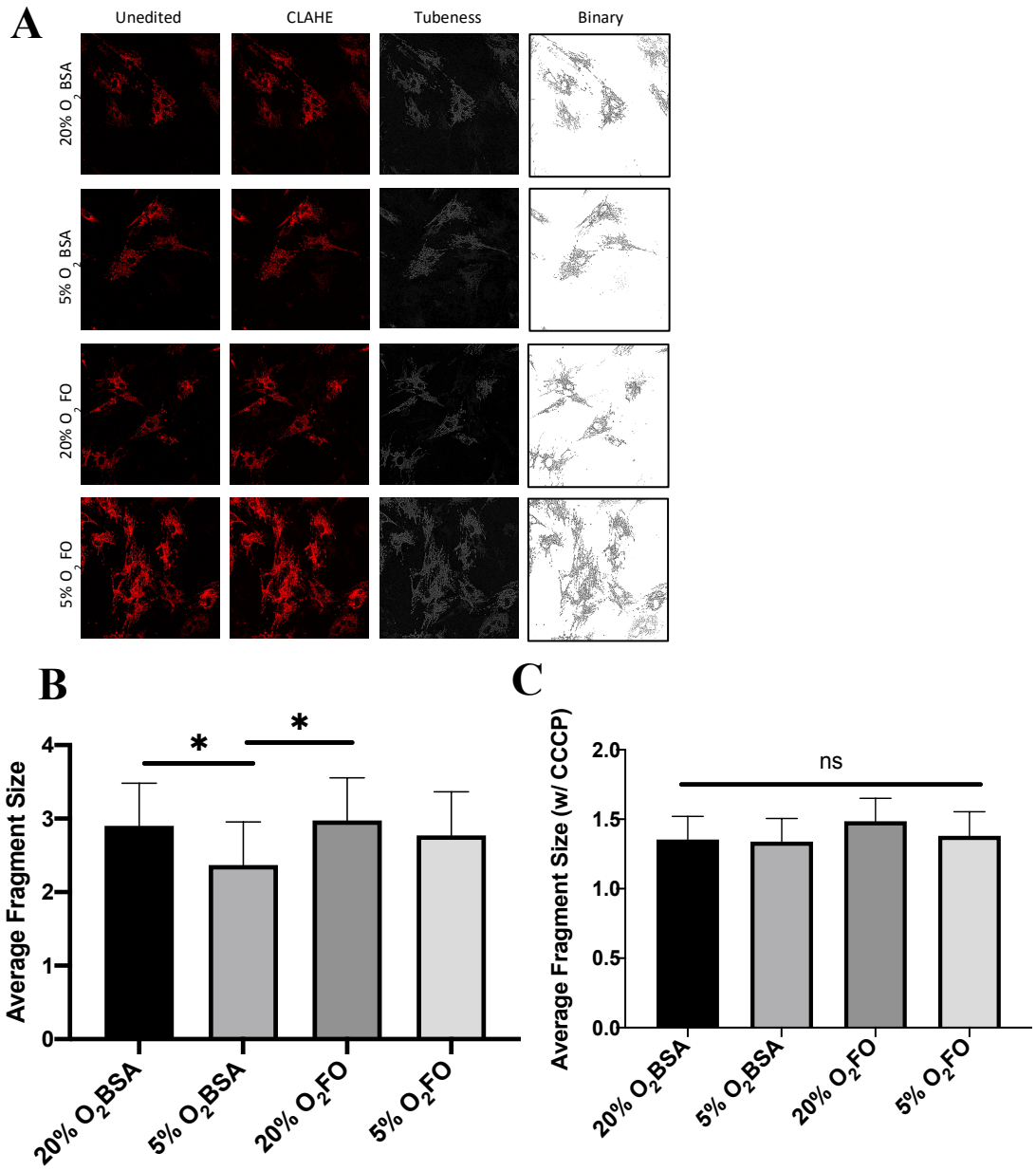

Figure 12: Effects of fish oil and hypoxia on mitochondrial fragment size: A: Representative images showing mitochondrial fragmentation. Images shown are unedited, CLAHE (enhanced local contrast), tubeness (particle separation), and binary (black and white). B: Average mitochondrial fragment size between cells treated without CCCP treatment. C: Average mitochondrial fragment size between cells treated with CCCP. BSA = bovine serum albumin control, FO = 0.03% fish oil (* $p < 0.05$, ns = not significant)

There was a decrease in mitochondrial fragment number in luteal cells treated with fish oil as compared to luteal cells treated with BSA, regardless of oxygen environment ($p < 0.01$, $p < 0.05$; Figure 13A). In CCCP treated cells, mitochondrial fragment number was decreased when treated with fish oil as compared to BSA, regardless of oxygen environment ($p < 0.01$, $p < 0.05$; Figure 13B).

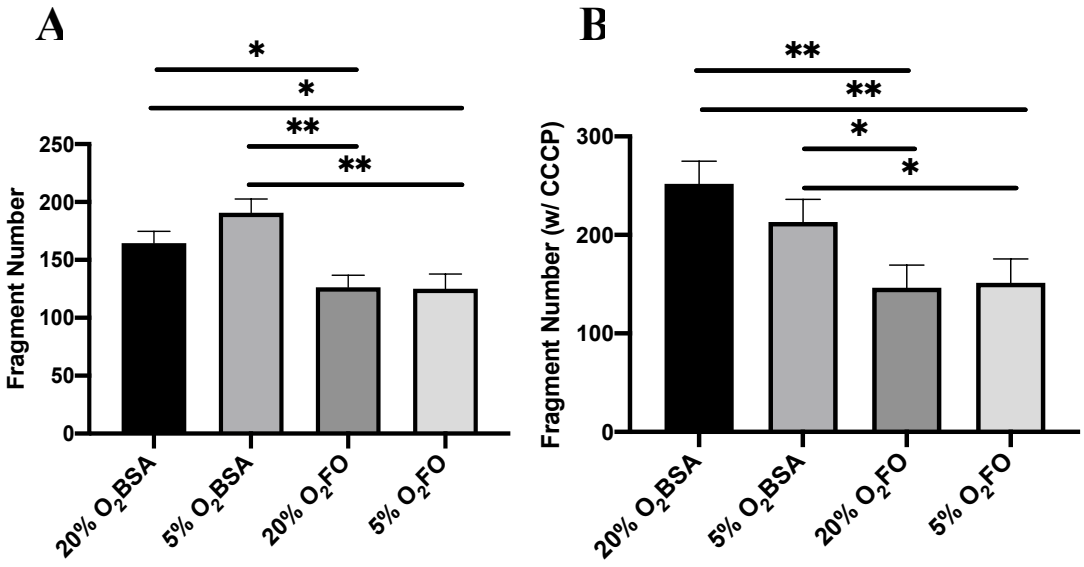

Figure 13: Effects of fish oil and hypoxia on mitochondrial fragment number. A: Mitochondrial fragment number between cells without CCCP treatment. B: Mitochondrial fragment number between cells with CCCP treatment. BSA = bovine serum albumin control, FO = 0.03% fish oil (* $p < 0.05$, ** $p < 0.01$)

Mitochondrial footprint was measured for each treatment group to determine overall organelle coverage. Mitochondrial footprint was higher in BSA treated luteal cells as compared to fish oil treated luteal cells, regardless of oxygen environment ($p < 0.01$; Figure 14A). Additionally, CCCP reduced mitochondrial footprint, however, mitochondrial footprint remained higher in BSA treated cells as compared to fish oil treated cells, regardless of CCCP treatment ($p < 0.01$, $p < 0.05$; Figure 14B).

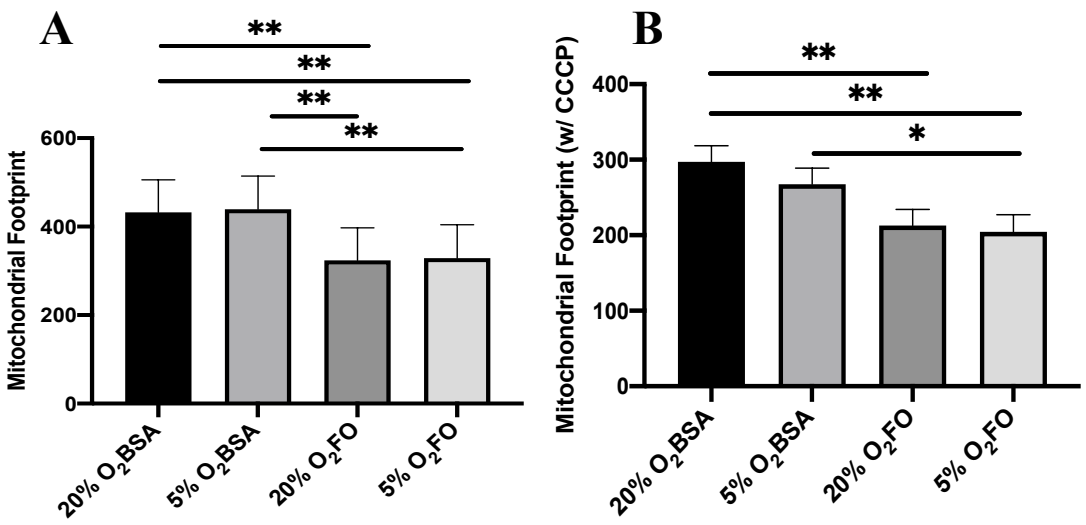

Figure 14: Effects of fish oil and hypoxia on mitochondrial footprint. A: Mitochondrial footprint without CCCP. B: Mitochondrial footprint with CCCP. BSA = bovine serum albumin control, FO = 0.03% fish oil (* $p < 0.05$, ** $p < 0.01$)

Mitochondrial morphology was further investigated using networking. Mitochondrial networking was used to determine number of mitochondrial branches and junctions. There was an increase in mitochondrial branch number for BSA treated luteal cells cultured in a normoxic environment as compared to BSA treated luteal cells cultured in a hypoxic environment ($p < 0.01$, $p < 0.05$; Figure 15B). Furthermore, there was an increase in mitochondrial branch number for BSA treated luteal cells cultured in a normoxic environment as compared to fish oil treated cells, regardless of oxygen environment ($p < 0.01$, $p < 0.05$; Figure 15B). Additionally, there was an increase in mitochondrial junction number for BSA treated cells cultured in normoxia, as compared to either BSA treated cells cultured in hypoxia or fish oil treated cells, regardless of oxygen environment ($p < 0.01$, $p < 0.05$; Figure 15C).

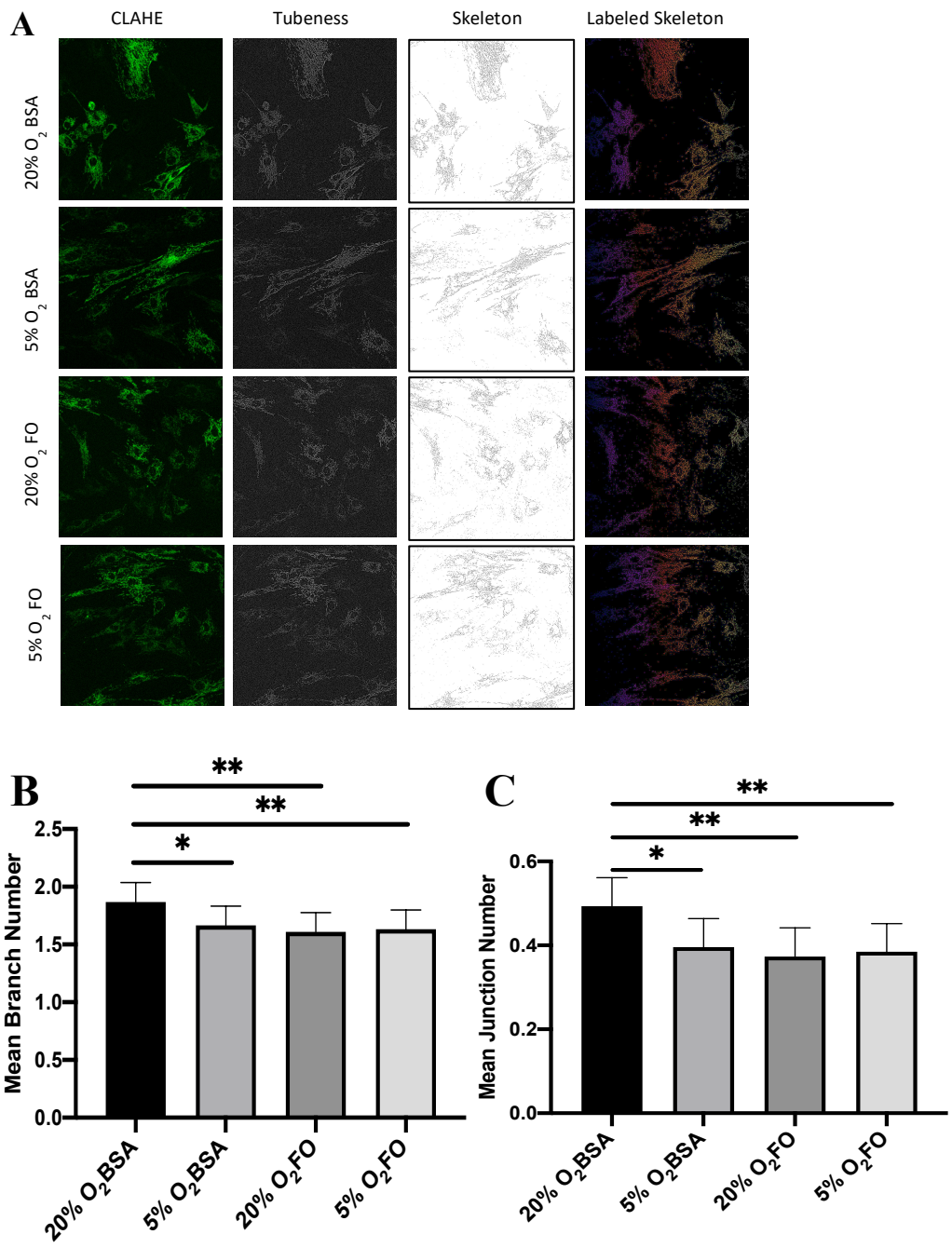

Figure 15: Effects of fish oil and hypoxia on mitochondrial branch number and mitochondrial junction number. A: Representative images showing morphological changes. Images shown are CLAHE (enhanced local contrast), tubeness (particle separation), skeleton (particle thickness of mitochondria) and labeled skeleton (colorized labeling of ROI; region of interest). B: Average mitochondrial branch number. C: Average mitochondrial junction number. BSA = bovine serum albumin control. FO = 0.03% fish oil (* $p < 0.05$, ** $p < 0.01$)

Effects of Hypoxia on Lipid Droplets

Lipid droplet number was greater in luteal cells at cultured in a hypoxic environment at 24 h, as compared to a normoxic environment ($p < 0.05$; Figure 16B). Additionally, lipid droplet size was greater in luteal cells cultured in a normoxic environment at 12 h, as compared to luteal cells cultured in a hypoxic environment ($p < 0.05$; Figure 16C).

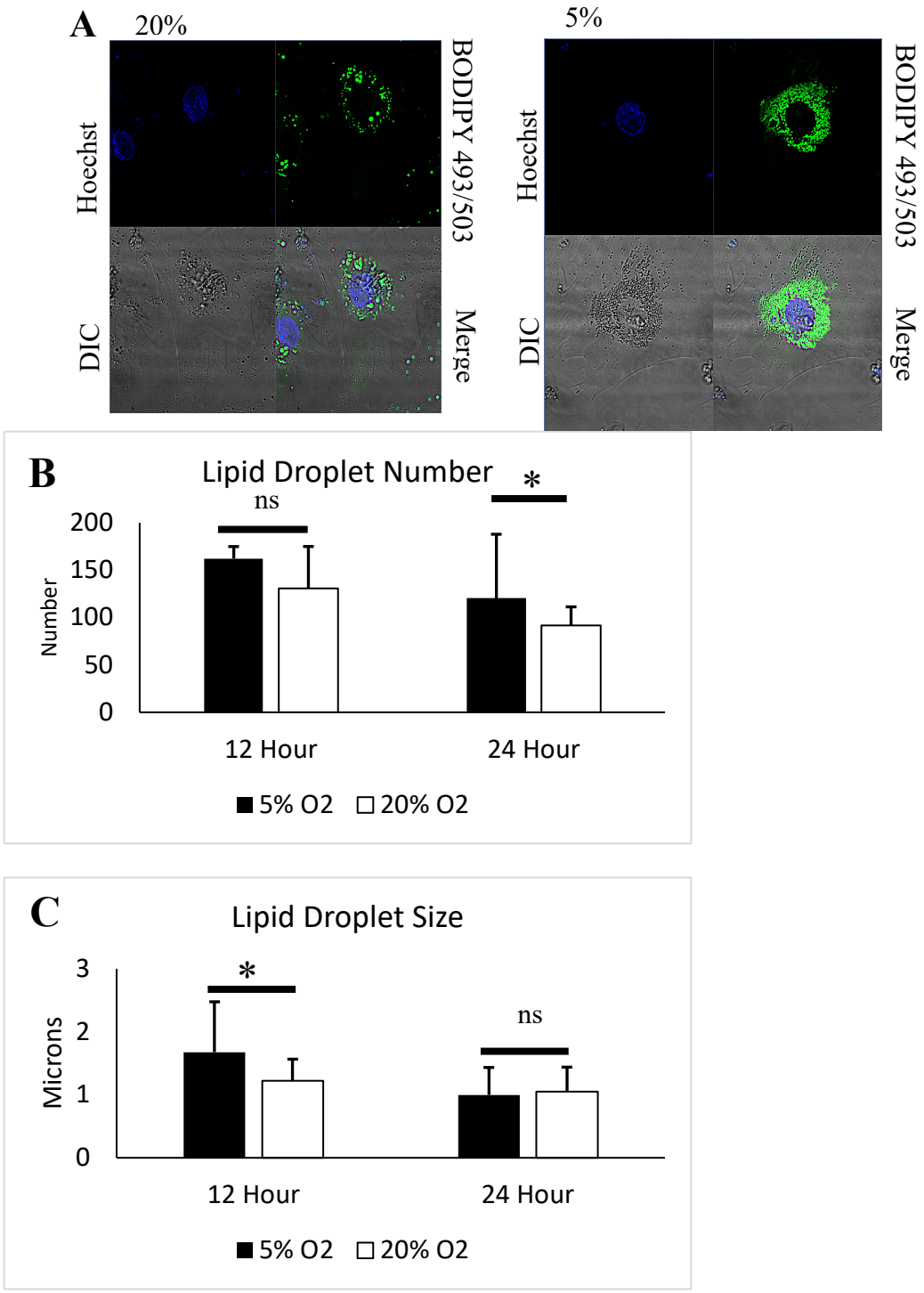

Figure 16: Effects of hypoxia on lipid droplet size and accumulation in bovine luteal cells. A: Representative images showing cell differences in lipid droplet size and number between hypoxia and normoxia. B: Lipid droplet number. C: Lipid droplet size. (* $p < 0.05$, ns = not significant)

CHAPTER IV

DISCUSSION

The objective of this study was to determine the effects of dietary supplementation of fish oil on PGF2a induced corpus luteum regression. Supplementation with fish byproduct in the form of fish oil or meal has been shown to prevent corpus luteum regression in the bovine, leading to increased progesterone synthesis (Mattos et al., 2002; Childs et al., 2008). It has been reported that regardless of corpus luteum regression, cows treated with PGF2a showed a significant decrease in steady-state mRNA for steroidogenic genes (Plewes et al., 2018). The purpose of this study was to determine if there was a rebound in steroidogenic gene mRNA following intrauterine infusions of PGF2a.

Previous literature shows that supplementation with fish byproduct leads to a significant increase in blood plasma EPA and DHA (Burns et al., 2003; White et al., 2012; Plewes et al., 2018; Moussavi et al., 2007), which is in alignment with our results. Cows receiving fish oil supplementation had increased plasma EPA and DHA beginning at day 35 of supplementation (Figure 4), which remained elevated compared to vegetable oil control cows for the remainder of the supplementation period. There were no significant weight changes between fish or vegetable oil supplemented cows at initial or final day of supplementation, which is in agreement with previous studies from our laboratory (White et al., 2012; Plewes et al., 2018). Therefore, changes in luteal

sensitivity to PGF2a are most likely due to increased plasma EPA and DHA from dietary supplementation.

In the present study, dietary supplementation did not have an effect on serum progesterone in cows treated with saline control (Figure 5A). These results are in alignment with previous literature indicating that fish oil or meal supplementation had minimal effects on luteal progesterone secretion (Moussavi et al., 2007; Mattos et al., 2002; Childs et al., 2008; Plewes et al., 2018; Wamsley et al., 2005; White et al., 2012). Intrauterine infusions of PGF2a at h 0 and 12 significantly decreased serum progesterone (Figure 5B), which resulted in functional regression for 43% of vegetable oil control cows, as opposed to only 25% of fish oil cows. This result is similar to previously reported literature (Plewes et al., 2018) where fish meal reduces corpus luteum sensitivity to PGF2a. Serum progesterone remained elevated for the remainder of the experimental collection period in cows that did not show a regressed corpus luteum.

Corpus luteum regression occurs in two separate ways, functional regression and structural regression (Nancarrow et al., 2973; Peterson et al., 1975; Juengel et al., 1993). Functional regression results in loss of progesterone synthesis whereas structural regression results in a decrease of gland mass. In cows that received intrauterine infusions of saline, there was no decrease in corpus luteum diameter regardless of supplementation (Figure 5B). Previous literature reports that fish oil or meal supplementation does not affect corpus luteum diameter (Plewes et al., 2018, Childs et al., 2008). Additionally, recent literature reports show that intrauterine infusions with PGF2a results in a significant decrease in corpus luteum diameter (Plewes et al., 2018). Surprisingly, there was no decrease in corpus luteum diameter in fish oil supplemented cows infused with

PGF2a that did not show functional regression (Figure 5B), indicating lack of both functional and structural regression. This result was not anticipated given that previous literature reports a decrease in corpus luteum diameter regardless of serum progesterone levels in response to fish meal supplementation (Plewes et al., 2018). One possible mechanism for this result would be increased membrane fluidity in response to fish oil supplementation, which subsequently decreases cell responsiveness to apoptotic factors recruited by PGF2a.

Progesterone biosynthesis is regulated by a set of key genes that are responsible for synthesis of steroid hormones (Zhang et al., 2010; Rekawiecki et al., 2008;). Reports in the literature show that steady-state mRNA for steroidogenic genes decreases in response to intrauterine infusions of PGF2a (Plewes et al., 2018; Atli et al., 2012). Steady-state mRNA for StAR, CYP11A1 and LDLR decreased following intrauterine infusions of PGF2a in cows that received vegetable oil supplementation (Figure 6A, B, C). In cows that received fish oil supplementation, steroidogenic mRNA decreased in response to intrauterine infusions of PGF2a (Figure 6A, B, C). However, unlike vegetable oil supplemented cows with a regressed corpus luteum wherein steady-state mRNA continued to decline, fish oil supplemented cows showed a stabilization in steroidogenic mRNA regardless of corpus luteum function (Figure 6A, B, C).

A recent report in the literature (Atli et al., 2012) shows that intrauterine infusions of PGF2a decrease luteal CYP11A1 protein abundance. Preliminary data from this study show that intrauterine infusions of PGF2a tended to decrease CYP11A1 protein abundance in vegetable oil supplemented cows with a regressed corpus luteum (Figure 7). However, CYP11A1 protein abundance remained unchanged following PGF2a

treatment in fish oil supplemented cows regardless of corpus luteum function (Figure 7). Thus, it appears that fish oil supplementation may mitigate PGF2a-induced luteal protein reduction.

Cholesterol is stored in steroidogenic tissues in the form of intracellular lipid droplets. These lipid droplets serve as a cholesterol-rich reservoir to maintain steroidogenic output. A recent report in the literature shows that fish oil supplementation increases lipid droplet size in mouse preadipocytes (Hsieh et al., 2019) and accumulation in rabbit hepatocytes (Zhu et al., 2015). In the present study, fish oil increased both lipid droplet size and accumulation in luteal tissue following approximately 75 days of supplementation (Figure 9). Additionally, fish oil increased lipid droplet accumulation in cultured bovine small luteal cells, but not lipid droplet size. These data demonstrate that fish oil supplementation either *in vitro* or *in vivo* affect lipid droplet dynamics. However, the mechanism by which alternation in lipid droplet dynamics affect PGF2a-induced corpus luteum regression warrant further investigation.

PGF2a has been reported to decrease blood during luteal regression (Ginther et al., 2007). This decrease in oxygen could potentially lead to hypoxia and affect mitochondrial function and subsequently progesterone production. Recent studies have shown that hypoxia decreases progesterone output in bovine luteal cells *in vitro* (Nishimura et al., 2008; Hasegawa et al., 2019). In the present study, hypoxia significantly reduced forskolin-induced progesterone production as compared to luteal cells cultured in a normoxic environment (Figure 10), which is in agreement with previous studies. It was hypothesized that fish oil supplementation would protect mitochondria and maintain progesterone production within a hypoxic environment.

However, progesterone production in fish oil-treated cells was similar to BSA-control treated cells during hypoxia (Figure 10). Although fish oil failed to improve progesterone production during hypoxia, it is possible fish oil may improve mitochondrial morphology and thereby become more resilient to apoptosis during hypoxia (Nishimura et al., 2008).

Mitochondrial fusion and fission are opposing processes. During hypoxia, mitochondria are likely to undergo fission, which subsequently leads to apoptosis (Zhang et al., 2018). Additionally, mitochondrial fusion is correlated with steroid biosynthesis (Duarte et al., 2012). It was hypothesized that fish oil treatment would reduce mitochondrial fission during hypoxia. In the present study, hypoxia resulted in decreased fragment size in BSA-treated luteal cells (Figure 12), indicating induction of mitochondrial fission (Zorov et al., 2019). However, there was no significant decrease in fragment size in fish oil-treated cells that were cultured in a hypoxic environment, as compared to cells cultured in a normoxic environment (Figure 12). This is interpreted as fish oil preventing mitochondrial fission during hypoxia. Additionally, mitochondrial fragment number was reduced in luteal cells treated with fish oil regardless of oxygen environment (Figure 13). Taken together, these data show that fish oil may improve mitochondrial morphology during hypoxia and thereby decreasing the likelihood of apoptosis.

Mitochondria are dynamic organelles capable of interactions between each other. This is often referred to as mitochondrial networking. Mitochondrial branching and junctions are indicative of multiple mitochondria grouping (or fusing) together to create more surface area that can readily be used for things such as shuttling of cholesterol and electron transport chain efficiency (Osellame et al., 2012). In the present study, fish oil

treatment had no effect on mitochondrial networking when cells were cultured in either normoxic or hypoxic conditions (Figure15). These data corroborate with the progesterone output during hypoxia.

Previous reports in the literature show that hypoxia induces lipid droplet accumulation (Gordon et al., 1977). In the present study, hypoxia increased lipid droplet accumulation in bovine luteal cells (Figure 16), which is in agreement with previous reports in the literature (Gordon et al., 1977). It is postulated that hypoxia induces lipid droplet accumulation with associated long-chain fatty acids that could be utilized by the mitochondria for beta-oxidation (Fuhrmann et al., 2019). In these studies, fish oil treatment affected lipid dynamics. Future studies are warranted to investigate lipid droplet dynamics in fish oil treated cells during hypoxia.

CHAPTER V

CONCLUSION

Early embryonic mortality is a major problem in the United States beef and dairy industries. This leads to a significant loss in meat and milk production. Therefore, preventing these losses will ultimately increase profitability for the American rancher and dairy farmer. The use of fish oil may be a novel method for improving reproductive performance.

Both literature and previous data from our laboratory show that omega-3 fatty acids in fish byproducts can be incorporated into blood plasma, which may be readily available for reproductive tissues, including the corpus luteum. The increase in availability of omega-3 fatty acids may allow for decreased luteal sensitivity to PGF2a. Here, it was shown that fish oil supplementation reduces luteal sensitivity to intrauterine infusions of PGF2a. However, the cellular and molecular mechanisms that lead to a reduction in luteal sensitivity are still largely unknown. These studies show that steady-state mRNA for key proteins that regulate steroidogenesis are initially reduced during the first 18 h following PGF2a, but then remain unchanged during the remaining 48 h. Protein abundance for CYP11A1 remain elevated in fish oil supplemented cows that retained a functional corpus luteum, but was reduced in vegetable oil supplemented cows with a regressed corpus luteum.

I*n vitro* experiments from this study show that fish oil treatment improves mitochondrial morphology during hypoxia. This may be a potential mechanism whereby fish oil protects the bovine corpus luteum during PGF2a-induced hypoxia.

It has been postulated that slow developing embryos fail to mitigate uterine PGF2a secretion during maternal recognition of pregnancy. Data from the current studies show that fish oil is luteal protective. Further studies are warranted to demine if fish oil supplementation improves pregnancy outcome in slow developing embryos.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE PROTOCOL

From:	Elaine.Kim@colostate.edu
To:	Jason.Bruemmer@ColoState.EDU; Terry.Engle@ColoState.EDU; Pat.d.Burns@colostate.edu
Cc:	Megan.Comisso@colostate.edu
Subject:	Approval of: AMENDMENT Protocol 16-6761AA, "Effect of omega-3 fatty acids on bovine luteal cell lipid microdomains and PGF2a Signaling"
Date:	Wednesday, September 26, 2018 3:10:41 PM

The IACUC has approved the AMENDMENT protocol with the following

details. Protocol ID: 16-6761AA
Principal Investigator: Bruemmer, Jason
Protocol Title: Effect of omega-3 fatty acids on bovine luteal cell lipid microdomains and PGF2a Signaling Approval Date: September 26, 2018

The official approval document can be accessed online under the "Event History" section of your "approved" protocol listing in eProtocol. Access eProtocol: https://csu.keyusa.net

If you see one of the following in the Subject line, here is a key to what it means:

NEW = Initial Approval
AMENDMENT = An amendment to a previously approved protocol
CONTINUING REVIEW = An annual renewal of a previously approved protocol
FINAL = Closure of a previously approved protocol